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(54) Title: GENE EXPRESSION VECTORS WHICH GENERATE AN ANTIGEN SPECIFIC IMMUNE RESPONSE AND METHODS OF USING THE SAME

(57) Abstract

The invention consists of recombinant gene expression vectors and vaccines useful in immunization of a host against an antigen and methods for use of such vectors and vaccines. In particular, the recombinant gene expression vectors of the invention are plasmids, cosmids or viruses which include non-coding, palindromic regions of single or double-stranded DNA or RNA polynucleotides which include at least one cytosine-guanine dinucleotide motif in each palindrome. These polynucleotide regions of each expression vector are immunostimulatory and serve as adjuvants to vaccination protocols against target antigens. Most preferably, the recombinant gene expression vectors of the invention are naked; i.e., non-viral vectors not associated with a delivery vehicle such as a liposome. The invention also includes live viral vaccines wherein the viruses include immunostimulatory polynucleotides of the invention. According to a preferred method of the invention, a target protein antigen is administered through its expression by a recombinant gene expression vector which contains the non-coding, immunostimulatory polynucleotides of the invention. In the most preferred embodiment of the method of the invention, the recombinant gene expression vector is administered to tissues of the host which contain a relatively high concentration of antigen presenting cells (e.g., skin or mucosa) compared to other host tissues.

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IMMUNOSTIMULATORY POLYNUCLEOTIDE/IMMUNOMODULATORY MOLECULE CONJUGATES

RELATED U.S. PATENT APPLICATIONS

This is a continuation-in-part and utility conversion of U.S. Provisional Patent Application Serial No. 60/028,118, filed October 11, 1996.

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FIELD OF THE INVENTION

The invention relates to compositions comprising an immunomodulatory molecule (IMM) including an antigen, conjugated to a polynucleotide that contains or consists of at least one immunostimulatory oligonucleotide (ISS-PN). It also relates to methods for modulating the immune response of a vertebrate host to an antigen.

HISTORY OF THE RELATED ART

Conventionally, immunization of a host against an antigen is accomplished by repeatedly vaccinating the host with the antigen. While most current vaccines elicit reasonable antibody responses, cellular responses (in particular, major histocompatibility complex (MHC) class I-restricted cytotoxic T cells) are generally absent or weak. For many infectious diseases, such as tuberculosis and malaria, humoral responses are of little protective value against infection.

Given the weak cellular immune response to protein antigens, modulation of the immune responses to these antigens has clear importance. The ability to modify immune responses to protein or peptide antigen has implications for tumor therapy, for the treatment of allergic disorders and for treatment of other conditions achievable through induction of a vigorous cellular immune response.

SUMMARY OF THE INVENTION

The present invention provides compositions comprising an ISS-PN which is conjugated to an IMM (which includes an antigen) to form ISS-PN/IMM conjugates. The ISS-PN/IMM conjugates of the invention are biological response modifiers in the sense that they modify the humoral and cellular immune response of a host to an antigen.

Specifically, the ISS-PN and IMM components of the ISS-PN/IMM conjugates synergistically boost the magnitude of the host immune response against an antigen to a level greater than the host immune response to either the IMM, antigen or ISS-PN alone. The ISS-PN/IMM conjugates also shift the host cellular immune response away from the helper T lymphocyte type 2 (Th2) phenotype toward a helper T lymphocyte type 1 (Th1) phenotype. These responses to ISS-PN/IMM conjugates are particularly acute during the important early phase of the host immune response to an antigen.

To these ends, ISS-PN/IMM conjugates are delivered by any route through which antigen-sensitized host tissues will be contacted with the ISS-PN/IMM conjugate. ISS-PN/IMM conjugates administered in this fashion boost both humoral (antibody) and cellular (Th1 type) immune responses of the host. Thus, use of the method to boost the immune responsiveness of a host to subsequent challenge by a sensitizing antigen without immunization avoids the risk of Th2-mediated, immunization-induced anaphylaxis by suppressing IgE production in response to the antigen challenge. An especially advantageous use for this aspect of the invention is treatment of localized allergic responses in target tissues where the allergens enter the body, such as the skin and mucosa.

Suppression of the Th2 phenotype according to the invention is also a useful in reducing antigen-stimulated IL-4 and IL-5 production. Thus, the invention encompasses delivery of ISS-PN/IMM conjugates to a host to suppress the Th2

phenotype associated with conventional antigen immunization (e.g., for vaccination or allergy immunotherapy).

The shift to a Th1 phenotype achieved according to the invention is accompanied by increased secretion of IFN α, β and γ, as well as IL-12 and IL-18. Each of these cytokines enhance the host's immune defenses against intracellular pathogens, such as viruses. Thus, the invention encompasses delivery of ISS-PN/IMM conjugates to a host to combat pathogenic infection.

Angiogenesis is also enhanced in the Th1 phenotype (ostensibly through stimulation by IL-12). Thus, the invention encompasses delivery of ISS-PN/IMM conjugates to a host to stimulate therapeutic angiogenesis to treat conditions in which localized blood flow plays a significant etiological role; e.g., retinopathies.

The ISS-PN/IMM conjugates of the invention comprise an IMM conjugated to a polynucleotide that includes, or consists of, at least one immunostimulatory oligonucleotide (ISS-ODN) moiety. The ISS-ODN moiety is a single- or double-stranded DNA or RNA oligonucleotide having at least 6 nucleotide bases which may include, or consist of, a modified oligonucleoside or a sequence of modified nucleosides.

The ISS-ODN moieties comprise, or may be flanked by, a CpG containing nucleotide sequence or a p(IC) nucleotide sequence, which may be palindromic. Where the oligonucleotide moiety comprises a CpG sequence, it may include a hexamer structure consisting of: 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'. Examples of such hexamer structures are AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

In one aspect of the invention, the ISS-PN consists of an ISS-ODN. Alternatively, the ISS-PN comprises an ISS-ODN.

Conjugates of the invention also include PN/IMM wherein the PN serves as a carrier to introduce the IMM antigen into MHC Class I processing pathways not normally stimulated by soluble antigen, but lacks ISS activity and therefore does not stimulate a Th1 phenotype immune response. Examples of such PN/IMM are those wherein the CpG motif is mutated, for example, to a GpG motif.

In one aspect of the invention, the IMM conjugate partner to the ISS-PN consists of an antigen. Such antigens are selected from the group of antigens consisting of proteins, peptides, glycoproteins, polysaccharides and gangliosides.

In another aspect of the invention, the IMM conjugate partner comprises an antigen and further comprises an immunostimulatory molecule selected from the group of such molecules consisting of adjuvants, hormones, growth factors, cytokines, chemokines, targeting protein ligands, and trans-activating factors.

In another aspect of the invention, the ISS-PN/IMM conjugate is modified for targeted delivery by, for example, attachment to a monoclonal antibody, receptor ligand and/or liposome.

Pharmaceutically acceptable compositions of ISS-PN/IMM conjugates are provided for use in practicing the methods of the invention. Where appropriate to the contemplated course of therapy, the ISS-PN/IMM conjugates may be administered with anti-inflammatory or immunotherapeutic agents. Thus, a particularly useful composition for use in practicing the method of the invention is one in which an anti-inflammatory agent (e.g., a glucocorticoid) is mixed with, or further conjugated to, an ISS-PN/IMM conjugate.

The ISS-PN/IMM conjugates can also be provided in the form of a kit comprising ISS-PN/IMM conjugates and any additional medicaments, as well as a device for delivery of the ISS-PN/IMM conjugates to a host tissue and reagents for determining the biological effect of the ISS-PN/IMM conjugates on a treated host.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a graph of data demonstrating the vigorous Th1-type immune response (as measured by production of IgG2a against an IMM antigen) stimulated by ISS-PN/IMM (1:5 ratio) in comparison to the levels of Th2-like responses stimulated by an ISS containing, antigen encoding plasmid (pACB-Z); the antigen alone (β-gal); the antigen mixed with an ISS (1:5 ratio); the antigen conjugated to a non-stimulatory PN (mISS conj; 1:5 ratio); the antigen in adjuvant (alum) and, for reference, the IgG2a levels in naive (unexposed) mice. The horizontal axis represents the levels (units/ml) of antibody; the vertical axis represents the number of weeks following primary antigen exposure.

FIGURE 2 is a graph of data demonstrating the levels of Th2-type immune responses (as measured by production of IgG1 against an IMM antigen) stimulated by an ISS containing, antigen encoding plasmid (pACB-Z); the antigen alone (β-gal); the antigen mixed with an ISS (1:5 ratio); the antigen conjugated to a non-stimulatory PN (mISS conj; 1:5 ratio); the antigen in adjuvant (alum) and, for reference, the IgG1 levels in naive (unexposed) mice, all as compared to the vigorous Th1-type immune response produced in mice immunized with ISS-PN/IMM (1:5 ratio). The horizontal axis represents the levels (units/ml) of antibody; the vertical axis represents the number of weeks following primary antigen exposure.

FIGURE 3 is a graph of data demonstrating the vigorous Th1-type immune response (as measured by production of IgG2a against an IMM antigen) stimulated by ISS-PN/IMM in comparison to the levels of Th2-like responses stimulated by the antigen alone (AgE) and antigen conjugated to a non-stimulatory PN (mISS conj). Antigen to PN ratios are all 1:5. The horizontal axis represents the levels (units/ml) of antibody; the vertical axis shows the levels at 4 weeks following primary antigen exposure (shaded bars) and at 2 weeks following secondary antigen challenge (solid bars).

FIGURE 4 is a graph of data demonstrating the levels of Th2-type immune responses (as measured by production of IgG1 against an IMM antigen) stimulated by the antigen alone (AgE) and antigen conjugated to a non-stimulatory PN (mISS conj) in comparison to the vigorous Th1-type immune response stimulated in ISS-PN/IMM immunized mice. Antigen to PN ratios are all 1:5. The horizontal axis represents the levels (units/ml) of antibody; the vertical axis shows the levels at 4 weeks following primary antigen exposure (shaded bars) and at 2 weeks following secondary antigen challenge (solid bars).

FIGURE 5 is a graph of data demonstrating suppression of Th2 associated anti-antigen (AgE) IgE production by ISS-PN/IMM in comparison to the levels of IgE production stimulated by the antigen alone (AgE) and the antigen conjugated to a non-stimulatory PN (mISS conj). Antigen to PN ratios are all 1:5. The horizontal axis represents the levels (counts per minute; cpm) of antibody; the vertical axis shows the levels at 4 weeks following primary antigen exposure (shaded bars) and at 2 weeks following secondary antigen challenge (solid bars).

FIGURE 6 is a graph of data demonstrating the high levels of Th1 associated interferon γ (IFNg) production stimulated by ISS-PN/IMM in comparison to the relatively low levels of the Th1 cytokine stimulated by an ISS containing, antigen encoding plasmid (pACB-Z); the antigen alone (β-gal); the antigen mixed with an ISS; the antigen conjugated to a non-stimulatory PN (mISS conj); the antigen in adjuvant (alum) and, for reference, the IFNg levels in naive (unexposed) mice. Antigen to PN ratios are all 1:5. The horizontal axis represents the levels (ng/ml) of cytokine; the vertical axis shows the levels of cytokine at 4 weeks following primary antigen exposure (shaded bars).

FIGURE 7 is a graph of data demonstrating the vigorous antigen-specific cytotoxic T lymphocyte (CTL) response stimulated by ISS-PN/IMM in comparison to the levels of CTL production stimulated by an ISS containing, antigen encoding plasmid (pACB-

Z); the antigen alone (β-gal); the antigen mixed with an ISS; the antigen conjugated to a non-stimulatory PN (mISS conj); the antigen in adjuvant (alum) and, for reference, the CTL levels in naive (unexposed) mice. Antigen to PN ratios are all 1:5. The horizontal axis represents the levels of antigen-specific cell lysis obtained (as a percentage of control; no antigen); the vertical axis shows the levels of CTL detected at different effector (antigen) to target ratios, from 0:1 to 10:1. The legend identifies how each cell population was treated.

DETAILED DESCRIPTION OF THE INVENTION

A. *Biological Activity of the ISS-PN/IMM Conjugates*

The immune response stimulated by the ISS-PN/IMM conjugates of the invention differs from the vertebrate immune response to conventional vaccination in both magnitude and quality. In the former respect, the host immune response to an antigen is boosted to a level greater than achieved on exposure to an ISS-PN or antigen administered alone or together in an unconjugated form. Thus, one surprising aspect of the invention is that conjugation of an ISS-PN to an antigen-containing IMM produces a synergism between the immunostimulatory activity of the ISS-PN and the immunomodulatory activity of the IMM that immunizes the host to the antigen more effectively than one would predict.

Advantageously, the immune response stimulated according to the invention differs from the immune response of vertebrates to conventional vaccination in that the latter develops in a Th2 phenotype while the former develops in a Th1 phenotype. In this regard, it is helpful to recall that CD4+ lymphocytes generally fall into one of two distinct subsets; i.e., the Th1 and Th2 cells. Th1 cells principally secrete IL-2, IFNγ and TNFβ (the latter two of which mediate macrophage activation and delayed type hypersensitivity) while Th2 cells principally secrete IL-4 (which stimulates production of IgE antibodies), IL-5 (which stimulates granulocyte infiltration of tissue), IL-6 and IL-10. These CD4+ subsets exert a negative influence on one another; i.e., secretion of Th1 lymphokines inhibits secretion of Th2 lymphokines and vice versa.

Factors believed to favor Th1 activation resemble those induced by viral infection and include intracellular pathogens, exposure to IFN-β, IFN-α, IFNγ, IL-12 and IL-18 and exposure to low doses of antigen. Th1 type immune responses also predominate in autoimmune disease. Factors believed to favor Th2 activation include exposure to IL-4 and IL-10, APC activity on the part of B lymphocytes and high doses of antigen.

Active Th1 (IFNγ) cells enhance cellular immunity and are therefore of particular value in responding to intracellular infections, while active Th2 cells enhance antibody production and are therefore of value in responding to extracellular infections (at the risk of anaphylactic events associated with IL-4 stimulated induction of IgE antibody production). Thus, the ability to shift host immune responses from the Th1 to the Th2 repertoire and vice versa has substantial clinical significance for controlling host immunity against antigen challenge (e.g., in infectious and allergic conditions).

To that end, the methods of the invention shift the host immune response to a sensitizing antigen toward a Th1 phenotype (Example I). Consequently, Th2 associated cytokine production and antigen stimulated production of IgE (Examples II and III) are suppressed, thereby reducing the host's risk of prolonged allergic inflammation and minimizing the risk of antigen-induced anaphylaxis. CTL production is also stimulated to a greater degree in animals treated according to the invention. Because CTL production is tied to antigen processing in Class I MHC pathways, increased CTL production can be produced from non-immunostimulatory PN/IMM as well as ISS-PN/IMM (Example IV).

Although the invention is not limited to any particular mechanism of action, it is conceivable that PN facilitate uptake of exogenous antigen by antigen presenting cells for presentation through host MHC Class I processing pathways not normally stimulated by soluble antigen. Thus, ISS-PN/IMM carry antigen into MHC Class I processing pathways (which may also be achieved by PN/IMM without ISS activity) then stimulate a cytokine cascade in a Th1 phenotype (as a result of ISS activity). Whatever the mechanism of action, use of ISS-PN/IMM to boost the host's immune responsiveness to a sensitizing antigen and shift the immune response toward a Th1 phenotype avoids the risk of immunization-induced anaphylaxis, suppresses IgE production in response to a sensitizing antigen and eliminates the need to identify the sensitizing antigen for use in immunization.

With reference to the invention, "boosting of immune responsiveness in a Th1 phenotype" in an ISS-PN/IMM treated host is evidenced by:

(1) a reduction in levels of IL-4 measured before and after antigen-challenge; or detection of lower (or even absent) levels of IL-4 in a treated host as compared to an antigen-primed, or primed and challenged, control;

(2) an increase in levels of IL-12, IL-18 and/or IFN (α, β or γ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN (α, β or γ) in an ISS-PN/IMM treated host as compared to an antigen-primed or, primed and challenged, control;

(3) IgG2a antibody production in a treated host; or

(4) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower (or even absent) levels of antigen-specific IgE in an ISS-PN/IMM treated host as compared to an antigen-primed, or primed and challenged, control.

Exemplary methods for determining such values are described further in the Examples.

Thus, the ISS-PN/IMM conjugates of the invention provide relatively safe, effective means of stimulating a robust immune response in a vertebrate host against any antigen.

B. *ISS-PN/IMM Conjugates: Structure and Preparation*

1. ISS-PN root structure

The ISS-ODN base of the ISS-PN/IMM conjugates of the invention includes an oligonucleotide, which may be a part of a larger nucleotide construct such as a

plasmid. The term "polynucleotide" therefore includes oligonucleotides, modified oligonucleotides and oligonucleosides, alone or as part of a larger construct. The polynucleotide may be single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA).

The polynucleotide portion can be linearly or circularly configured, or the oligonucleotide portion can contain both linear and circular segments. Modifications of oligonucleotides include, but are not limited to, modifications of the 3'OH or 5'OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group.

The oligonucleotide base of ISS-PN/IMM conjugates may comprise ribonucleotides (containing ribose as the only or principal sugar component), deoxyribonucleotides deoxyribose as the principal sugar component), or in accordance with established state-of-the-art modified sugars or sugar analogs may be incorporated in the oligonucleotide of the present invention. Thus, in addition to ribose and deoxyribose, the sugar moiety may be pentose, deoxypentose, hexose, deoxyhexos, glucose, arabinose, xylose, lyxose, and a sugar "analog" cyclopentyl group. The sugar may be in pyranosyl or in a furanosyl form. In the modified oligonucleotides of the present invention the sugar moiety is preferably the furanoside of ribose, deoxyribose, arabinose or 2'-0-methylribose, and the sugar may be attached to the respective heterocyclic bases either in I or J anomeric configuration. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) per se is known, and need not be described here, except to the extent such preparation may pertain to any specific example.

The phosphorous derivative (or modified phosphate group) which may be attached to the sugar or sugar analog moiety in the modified oligonucleotides of the present invention may be a monophosphate, diphosphate, triphosphate, alkylphosphate,

alkanephosphate, phosphoronthioate, phosphorodithioate or the like. The preparation of the above-noted phosphate analogs, and their incorporation into nucleotides, modified nucleotides and oligonucleotides, per se, is also known and need not be described here.

The heterocyclic bases, or nucleic acid bases which are incorporated in the oligonucleotide base of the ISS-PN/IMM conjugates may be the naturally occurring principal purine and pyrimidine bases, (namely uracil or thymine, cytosine, adenine and guanine, as mentioned above), as well as naturally occurring and synthetic modifications of said principal bases. Those skilled in the art will recognize that a large number of "synthetic" non-natural nucleosides comprising various heterocyclic bases and various sugar moieties (and sugar analogs) have become available in the prior art, such that oligonucleotide base of the ISS-PN/IMM conjugates may include one or several heterocyclic bases other than the principal five base components of naturally occurring nucleic acids. Preferably, however, the heterocyclic base in the oligonucleotide base of the ISS-PN/IMM conjugates is selected form uracil-5-yl, cytosin-5-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl, guanin-8-yl, 4-aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrolo [2,3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the oligonucleotides via the 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via the 7-position and the pyrazolopyrimidines via the 1-position.

Structurally, the root oligonucleotide of the ISS-PN component of ISS-PN/IMM is a non-coding sequence which may include at least one unmethylated CpG motif. The relative position of any CpG sequence in ISS-PN with immunostimulatory activity in certain mammalian species (e.g., rodents) is 5'-CG-3' (i.e., the C is in the 5' position with respect to the G in the 3' position). PN/IMM can be conveniently obtained by substituting the cytosine in the CpG dinucleotide with another nucleotide; a particularly useful substitution is with a guanine to form GpG dinucleotide containing PN.

Some oligonucleotide ISS (ISS-ODN) are known. In such ISS-ODN, the CpG motif is flanked by at least two purine nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (5'-Purine-Purine-[C]-[G]-Pyrimidine-Pyrimidine-3'). CpG motif-containing ISS-ODN are believed to stimulate B lymphocyte proliferation (see, e.g., Krieg, *et al.*, *Nature*, 374:546-549, 1995).

The core hexamer structure of the foregoing ISS-PN may be flanked upstream and/or downstream by any number or composition of nucleotides or nucleosides. However, ISS-PN are at least 6 bases in length, and preferably are between 6 and 200 bases in length, to enhance uptake of the ISS-PN/IMM into target tissues. Those of ordinary skill in the art will be familiar with, or can readily identify, reported nucleotide sequences of known ISS-ODN for reference in preparing ISS-PN. For ease of reference in this regard, the following sources are especially helpful:

Yamamoto, *et al.*, *Microbiol.Immunol.*, 36:983 (1992)
Ballas, *et al.*, *J.Immunol.*, 157:1840 (1996)
Klinman, *et al.*, *J.Immunol.*, 158:3635 (1997)
Sato, *et al.*, *Science*, 273:352 (1996)

Each of these articles are incorporated herein by reference for the purpose of illustrating the level of knowledge in the art concerning the nucleotide composition of known ISS-ODN .

In particular, ISS-PN and PN useful in the invention include those which have the following hexameric nucleotide sequences:

1. For ISS-PN, hexamers having "CpG" motifs or, for PN, hexamers having XpY motifs, where X cannot be C if Y is G and vice-versa; and,

2. Inosine and/or uracil substitutions for nucleotides in the foregoing hexamer sequences for use as RNA ISS-ODN.

For example, DNA based ISS-PN useful in the invention include those which have the following hexameric nucleotide sequences:

AACGTT, AGCGTC, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AGCGCT, GACGCT, GGCGCT, TTCGAA, GGCGTT and AACGCC (respectively, SEQ.ID.Nos. 1-18).

RNA based ISS-PN useful in the invention include those which have the following hexameric nucleotide sequences:

AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC, GACGCU, GACGCpI, GACGCpC, GACGUU, GACGpI, GACGpC, GACGUC, GACGpI, GACGpC, and poly(I•C) (respectively, SEQ.ID.Nos. 19-33).

The ISS-PN may or may not include palindromic regions. If present, a palindrome may extend only to a CpG motif, if present, in the core hexamer sequence, or may encompass more of the hexamer sequence as well as flanking nucleotide sequences.

In addition, backbone phosphate group modifications (e.g., methylphosphonate, phosphorothioate, phosphoroamidate and phosphorodithioate internucleotide linkages) can confer anti-microbial activity on the ISS-PN and enhance their stability *in vivo*, making them particularly useful in therapeutic applications. A particularly useful phosphate group modification is the conversion to the phosphorothioate or phosphorodithioate forms of ISS-PN. In addition to their potentially anti-microbial

properties, phosphorothioates and phosphorodithioates are more resistant to degradation *in vivo* than their unmodified oligonucleotide counterparts, making the ISS-PN/IMM of the invention more available to the host.

2. IMM conjugate partners.

The oligonucleotide base of the ISS-PN/IMM conjugate is conjugated to an IMM which includes an antigen and may further include an immunomodulatory agent. An "antigen" is a substance that is recognized and bound specifically by an antibody or by a T cell antigen receptor. Antigens can include peptides, proteins, glycoproteins and polysaccharides, including portions thereof and combinations thereof. The antigens can be those found in nature or can be synthetic.

The term "immunomodulatory" as used herein includes immunostimulatory as well as immunosuppressive effects. Immunostimulatory effects include, but are not limited to, those that directly or indirectly enhance cellular or humoral immune responses. Examples of immunostimulatory effects include, but are not limited to, increased antigen-specific antibody production; activation or proliferation of a lymphocyte population such as NK cells, CD4+ T lymphocytes, CD8+ T lymphocytes, macrophages and the like; as well as increased synthesis of Th1 associated immunostimulatory cytokines including, but not limited to, IL-6, IL-12, IL-18, IFN-α,β and γ, TNF-α and the like. Immunosuppressive effects include those that directly or indirectly decrease cellular or humoral immune responses.

Examples of immunosuppressive effects include, but are not limited to, a reduction in antigen- specific antibody production such as reduced IgE production; activation of lymphocyte or other cell populations that have immunosuppressive activities such as those that result in immune tolerance; and increased synthesis of cytokines that have suppressive effects toward certain cellular functions. One example of this is IFN-γ, which can block IL-4 induced class switch to IgE and IgG1, thereby reducing the

levels of these antibody subclasses.

Thus, an "immunomodulatory agent" suitable for use as conjugate partners for ISS-PN/IMM can be a peptide, such as an antigen or cytokine. Where the ISS-PN/IMM conjugate partner is a peptide, suitable peptides include purified native peptides, synthetic peptides, recombinant proteins, crude protein extracts, attenuated or inactivated viruses, cells, micro-organisms, or fragments of such peptides.

Protein antigens that can serve as IMM conjugate partners include antigens from a wide variety of sources, including allergens such as plant pollens, dust mite proteins, animal dander, saliva, and fungal spores as well as infectious microorganims. Examples of the latter include attenuated or inactivated viruses such as HIV-1, HIV-2, hepatitis, herpes simplex, rotavirus, polio virus, measles virus, human and bovine papilloma virus, and slow brain viruses. For immunization against tumor formation, the conjugate can include tumor cells (live or irradiated), tumor cell extracts, or protein subunits of tumor antigens. Vaccines for immuno-based contraception can be formed by including sperm proteins as the peptide portion of the conjugate.

Among the suitable cytokines for use as components of IMM conjugate partners are the interleukins (IL-1, IL-2, IL-3, etc.), interferons (e.g., IFN-α, IFN-β, IFN-γ), erythropoietin, colony stimulating factors (e.g., G-CSF, M-CSF, GM-CSF) and TNF-α.

IMM conjugate partners can also include amino acid sequences that mediate protein binding to a specific receptor or that mediate targeting to a specific cell type or tissue. Examples include, but are not limited to, antibodies or antibody fragments; peptide hormones such as human growth hormone; and enzymes. Co-stimulatory molecules such as B7 (CD80), trans-activating proteins such as transcription factors, chemokines such as macrophage chemotactic protein (MCP) and other chemoattractant or chemotactic peptides are also useful peptide-based conjugate partners.

More specifically, suitable antigens for use as ISS-PN/IMM conjugate partners include any molecule capable of being conjugated to an oligonucleotide and eliciting a B cell or T cell antigen-specific response. Preferably, antigens elicit an antibody response specific for the antigen. A wide variety of molecules are antigens. These include, but are not limited to, sugars, lipids, autacoids and hormones, as well as macromolecules such as complex carbohydrates, and phospholipids. Small molecules may need to be haptenized in order to be rendered antigenic.

Preferably the antigens are peptides, polysaccharides (such as the capsular polysaccharides used in *Haemophilus influenza* vaccines), gangliosides and glycoproteins. The antigen may be an intact antigen or T cell epitope(s) of an antigen. These can be obtained through several methods known in the art, including isolation and synthesis using chemical and enzymatic methods. In certain cases, such as for many sterols fatty acids and phospholipids, the antigenic portions are commercially available.

Many antigenic peptides and proteins are known in, and available to the art; others can be identified using conventional techniques. Examples of known antigens include, but are not limited to :

a. Allergens such as reactive major dust mite allergens *Der pI* and *Der pII* (*see,* Chua, *et al., J.Exp.Med.*, 167:175-182, 1988; and, Chua, *et al., Int.Arch.Allergy Appl. Immunol.*, 91:124-129, 1990), T cell epitope peptides of the *Der pII* allergen (*see*, Joost van Neerven, *et al., J.Immunol.*, 151:2326-2335, 1993), the highly abundant Antigen E (*Amb aI*) ragweed pollen allergen (*see,* Rafnar, *et al., J.Biol.Chem.*, 266:1229-1236, 1991), phospholipase A_2 (bee venom) allergen and T cell epitopes therein (*see,* Dhillon, *et al., J.Allergy Clin.Immunol.*, __:42-__, 1992), white birch pollen (*Betvl)* (*see,* Breiteneder, *et al., EMBO,* 8:1935-1938, 1989), the *Fel dI* major domestic cat allergen (*see,* Rogers, *et al., Mol.Immunol.*, 30:559-568, 1993), tree pollen (*see*, Elsayed et al., *Scand. J. Clin. Lab. Invest. Suppl.*, 204:17-31,

1991) and grass pollen (*see*, Malley, *J. Reprod. Immunol.*, 16:173-86, 1989).

b. Live, attenuated and inactivated microorganisms such as inactivated polio virus (Jiang *et al.*, *J. Biol. Stand.*, 14:103-9, 1986), attenuated strains of Hepatitis A virus (Bradley *et al.*, *J. Med. Virol.*, 14:373-86, 1984), attenuated measles virus (James *et al.*, *N. Engl. J. Med.*, 332:1262-6, 1995) and epitopes of pertussis virus (e.g., ACEL-IMUNE® acellular DTP, Wyeth-Lederle Vaccines and Pediatrics).

c. Contraceptive antigens such as human sperm protein (Lea *et al.*, *Biochim. Biophys. Acta*, 1307:263, 1996).

The published sequence data and methods for isolation and synthesis of the antigens described in these articles are incorporated herein by this reference to illustrate knowledge in the art regarding useful antigen sources. Those of ordinary skill in the art will be familiar with, or can readily ascertain, the identity of other useful antigens for use as ISS-PN/IMM conjugate partners.

Particularly useful immunostimulatory peptides for inclusion in IMM are those which stimulate Th1 immune responses, such as IL-12 (Bliss, *et al.*, *J.Immunol.*, 156:887-894, 1996), IL-18, INF-α,β and γ or TGF-α. Conjugation of adjuvants (such as keyhole limpet hemocyanin, KLH) to the ISS-PN/IMM conjugate can further enhance the activity of the ISS-PN/IMM conjugates of the invention.

Other useful adjuvants include cholera toxin, procholeragenoid, cholera toxin B subunit and fungal polysaccharides including, but not limited to, schizophyllan, muramyl dipeptide, muramyl dipeptide derivatives, phorbol esters, microspheres, non-Helicobacter pylori bacterial lysates, labile toxin of Escherichia coli, block polymers, saponins, and ISCOMs. For additional adjuvants, those of ordinary skill in the art may also refer to, for example, Azuma, I., "Synthetic Immunoadjuvants: Application to Non-Specific Host Stimulation and Potentiation of Vaccine Immunogenicity"

Vaccine, vol. 10, 1000 (1992); Pockley, A.G. & Montgomery, P.C., "In vivo Adjuvant Effect of Interleukins 5 and 6 on Rat Tear IgA Antibody Responses" *Immunology*, vol. 73, 19-23 (1991); Adam, A. & Lederer, E. "Muramyl peptides as Immunomodulators" ISI ATLAS OF SCIENCE 205 (1988); Clements, J.D., et al. "Adjuvant Activity of Escherichia coli Heat-labile Enterotoxin and Effect on the Induction of Oral Tolerance in Mice to Unrelated Protein Antigens" *Vaccine*, vol. 6, 269 (1988); Ben Ahmeida, E.T.S., *et al.* "Immunopotentiation of Local and Systemic Humoral Immune Responses by ISCOMs, Liposomes and FCA: Role in Protection Against Influenza A in Mice" *Vaccine*, vol. 11, 1302 (1993); and Gupta, R.K. *et al.* "Adjuvants -- A Balance Between Toxicity and Adjuvanticity" *Vaccine*, vol. 11, 290-308 (1993).

Those of ordinary skill in the art will appreciate that non-antigen components of IMM described above can also be administered in unconjugated form with an ISS-PN/IMM (antigen only) conjugate. Thus, the co-administration of such components is encompassed by the invention.

C. *Synthesis of Polynucleotide Conjugates*

1. Polynucleotide portion

ISS-PN can be synthesized using techniques and nucleic acid synthesis equipment which are well-known in the art. For reference in this regard, see, e.g., Ausubel, *et al.*, *Current Protocols in Molecular Biology*, Chs. 2 and 4 (Wiley Interscience, 1989); Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., New York, 1982); U.S. Patent No. 4,458,066 and U.S. Patent No. 4,650,675. When assembled enzymatically, the individual units can be ligated with a ligase such as T4 DNA or RNA ligase as described in, for example, U.S. Patent No. 5,124,246. Oligonucleotide degradation could be accomplished through the exposure of an oligonucleotide to a nuclease, as exemplified in U.S. Patent No. 4,650,675. These references are incorporated herein by reference for the sole purpose of demonstrating knowledge in the art concerning production of synthetic polynucleotides. Because the

ISS-PN is non-coding, there is no concern about maintaining an open reading frame during synthesis.

Alternatively, ISS-PN may be isolated from microbial species (especially mycobacteria) using techniques well-known in the art, such as nucleic acid hybridization. Preferably, such isolated ISS-PN will be purified to a substantially pure state; i.e., to be free of endogenous contaminants, such as lipopolysaccharides. ISS-PN isolated as part of a larger polynucleotide can be reduced to the desired length by techniques well known in the art, such as by endonuclease digestion. Those of ordinary skill in the art will be familiar with, or can readily ascertain, techniques suitable for isolation, purification and digestion of polynucleotides to obtain ISS-PN of potential use in the invention.

Circular ISS-PN can be isolated, synthesized through recombinant methods, or chemically synthesized. Where the circular ISS-PN is obtained through isolation or through recombinant methods, the ISS-PN will preferably be a plasmid. The chemical synthesis of smaller circular oligonucleotides can be performed using literature methods (Gao et al., Nucleic Acids Res. (1995) 23:2025-9; Wang et al., Nucleic Acids Res. (1994) 22:2326-33).

The ISS-PN can also contain modified oligonucleotides. These modified oligonucleotides can be synthesized using standard chemical transformations. The efficient solid-support based construction of methylphosphonates has been described. Agrawal et al. (19) Tet. Lett. 28:3539-3542. The synthesis of other phosphorous based modified oligonucleotides, such as phosphotriesters (Miller et al. JACS 93, 6657-6665), phosphoramidates (Jager et al, Biochemistry 27, 7247-7246), and phosphorodithioates (U.S. Patent No. 5,453,496) has also been described. Other non-phosphorous based modified oligonucleotides can also be used (Stirchak et al., Nucleic Acids Res. 17, 6129-6141).

The preparation of base-modified nucleosides, and the synthesis of modified oligonucleotides using said base-modified nucleosides as precursors, has been described, for example, in U.S. Patents 4,910,300, 4,948,882, and 5,093,232. These base-modified nucleosides have been designed so that they can be incorporated by chemical synthesis into either terminal or internal positions of an oligonucleotide. Such base-modified nucleosides, present at either terminal or internal positions of an oligonucleotide, can serve as sites for attachment of a peptide or other antigen. Nucleosides modified in their sugar moiety have also bee described (e.g., U.S. Patents 4,849,513, 5,015,733, 5,118,800, 5,118,802) and can be used similarly.

The techniques for making phosphate group modifications to oligonucleotides are known in the art and do not require detailed explanation. For review of one such useful technique, the an intermediate phosphate triester for the target oligonucleotide product is prepared and oxidized to the naturally occurring phosphate triester with aqueous iodine or with other agents, such as anhydrous amines. The resulting oligonucleotide phosphoramidates can be treated with sulfur to yield phophorothioates. The same general technique (excepting the sulfur treatment step) can be applied to yield methylphosphoamidites from methylphosphonates. For more details concerning phosphate group modification techniques, those of ordinary skill in the art may wish to consult U.S. Patent Nos. 4,425,732; 4,458,066; 5,218,103 and 5,453,496, as well as *Tetrahedron Lett.* at 21:4149 (1995), 7:5575 (1986), 25:1437 (1984) and *Journal Am.ChemSoc.*, 93:6657 (1987), the disclosures of which are incorporated herein for the sole purpose of illustrating the standard level of knowledge in the art concerning preparation of these compounds.

2. Linking the PN component to the IMM component

The ISS-PN component can be linked to the IMM portion of the conjugate in a variety of ways. The link can be made at the 3' or 5' end of the ISS-PN, or to a suitably modified base at an internal position in the PN. If the peptide contains a suitable

reactive group (e.g., an N-hydroxysuccinimide ester) it can be reacted directly with the N^4 amino group of cytosine residues. Depending on the number and location of cytosine residues in the ISS-PN, specific labeling at one or more residues can be achieved.

Alternatively, modified oligonucleosides, such as are known in the art, can be incorporated at either terminus, or at internal positions in the ISS-PN. These can contain blocked functional groups which, when deblocked, are reactive with a variety of functional groups which can be present on, or attached to, a peptide of interest.

The IMM portion of the conjugate can be attached to the 3'-end of the ISS-PN through solid support chemistry. For example, the ISS-PN portion can be added to a polypeptide portion that has been pre-synthesized on a support (Haralambidis et al., Nucleic Acids Res. (1990) 18:493-99; Haralambidis et al., Nucleic Acids Res. (1990) 18:501-505). Alternatively, the PN can be synthesized such that it is connected to a solid support through a cleavable linker extending from the 3'-end. Upon chemical cleavage of the ISS-PN from the support, a terminal thiol group is left at the 3'-end of the ISS-PN (Zuckermann et al., Nucleic Acids Res. (1987) 15:5305-5321; Corey et al., (1987) Science 238:1401-1403), or a terminal amine group is left at the 3'-end of the PN (Nelson et al., Nucleic Acids Res. (1989) 17:1781-94). Conjugation of the amino-modified PN to amino groups of the peptide can be performed as described in Benoit et al., Neuromethods (1987) 6:43-72. Conjugation of the thiol-modified ISS-PN to carboxyl groups of the peptide can be performed as described in Sinah et al., Oligonucleotide Analogues: A Practical Approach (1991) IRL Press.

The IMM portion of the conjugate can be attached to the 5'-end of the ISS-PN through an amine, thiol, or carboxyl group that has been incorporated into the ISS-PN during its synthesis. Preferably, while the ISS- PN is fixed to the solid support, a linking group comprising a protected amine, thiol, or carboxyl at one end, and a phosphoramidite at the other, is covalently attached to the 5'-hydroxyl (Agrawal et al.,

Nucleic Acids Res. (1986) 14:6227-6245; Connolly, Nucleic Acids Res. (1985) 13:4485-4502; Coull et al., Tetrahedron Lett. (1986) 27:3991-3994; Kremsky et al., Nucleic Acids Res. (1987) 15:2891-2909; Connolly, Nucleic Acids Res. (1987) 15:3131-3139; Bischoff et al., Anal. Biochem. (1987) 164:336-344; Blanks et al., Nucleic Acids Res. (1988) 16:10283-10299; U.S. Patent Nos. 4,849,513, 5,015,733, 5,118,800, and 5,118,802). Subsequent to deprotection, the latent amine, thiol, and carboxyl functionalities can be used to covalently attach the PN to a peptide (Benoit et al., Neuromethods (1987) 6:43-72; Sinah et al., Oligonucleotide Analogues: A Practical Approach (1991) IRL Press).

A peptide portion can be attached to a modified cytosine or uracil at any position in the ISS-PN. The incorporation of a "linker arm" possessing a latent reactive functionality, such as an amine or carboxyl group, at C-5 of the modified base provides a handle for the peptide linkage (Ruth, 4th Annual Congress for Recombinant DNA Research, p. 123).

The linkage of the ISS-PN to a peptide can also be formed through a high-affinity, non-covalent interaction such as a biotin-streptavidin complex. A biotinyl group can be attached, for example, to a modified base of an oligonucleotide (Roget et al., Nucleic Acids Res. (1989) 17:7643-7651). Incorporation of a streptavidin moiety into the peptide portion allows formation of a non-covalently bound complex of the streptavidin conjugated peptide and the biotinylated PN.

The linkage of the ISS-PN to a lipid can be formed using standard methods. These methods include, but are not limited to, the synthesis of oligonucleotide-phospholipid conjugates (Yanagawa et al., Nucleic Acids Symp. Ser. (1988) 19:189-92), oligonucleotide-fatty acid conjugates (Grabarek et al., Anal. Biochem. (1990) 185:131-35; Staros et al., Anal. Biochem. (1986) 156:220-22), and oligonucleotide-sterol conjugates (Boujrad et al., Proc. Natl. Acad. Sci. USA (1993) 90:5728-31).

The linkage of the ISS-PN to a oligosaccharide can be formed using standard known

methods. These methods include, but are not limited to, the synthesis of oligonucleotide-oligosaccharide conjugates, wherein the oligosaccharide is a moiety of an immunoglobulin (O'Shannessy et al., J. Applied Biochem. (1985) 7:347-55).

Adjuvants and cytokines may also be genetically or chemically linked to the ISS-ODN conjugates. Examples of this type of fusion peptide are known to those skilled in the art and can also be found in Czerkinsky *et al.*, *Infect. Immun.*, 57: 1072-77 (1989); Nashar *et al.*, *Vaccine*, 11: 235-40 (1993); and Dertzbaugh and Elson, *Infect. Immun.*, 61: 48-55 (1993).

The linkage of a circular ISS-PN to an IMM can be formed in several ways. Where the circular PN is synthesized using recombinant or chemical methods, a modified nucleoside (Ruth, in *Oligonucleotides and Analogues: A Practical Approach* (1991) IRL Press). Standard linking technology can then be used to connect the circular ISS-PN to the antigen or immunostimulatory peptide (Goodchild, Bioconjugate Chem. (1990) 1: 165). Where the circular ISS-PN is isolated, or synthesized using recombinant or chemical methods, the linkage can be formed by chemically activating, or photoactivating, a reactive group (e.g. carbene, radical) that has been incorporated into the antigen or immunostimulatory peptide.

Additional methods for the attachment of peptides and other molecules to ISS-PNs can be found in C. Kessler: *Nonradioactive labeling methods for nucleic acids* in L.J. Kricka (ed.) "Nonisotopic DNA Probe Techniques," Academic Press 1992 and in Geoghegan and Stroh, *Bioconjug. Chem.*, 3:138-146, 1992.

D. *Methods and Routes for Administration of ISS-PN/IMM to a Host*

1. Drug delivery

The ISS-PN/IMM of the invention are administered to a host using any available method and route suitable for drug delivery, including *ex vivo* methods (e.g., delivery

of cells incubated or transfected with an ISS-PN/IMM) as well as systemic or localized routes. However, those of ordinary skill in the art will appreciate that methods and localized routes which direct the ISS-PN/IMM into antigen-sensitized tissue will be preferred in most circumstances to systemic routes of administration, both for immediacy of therapeutic effect and avoidance of *in vivo* degradation.

The entrance point for many exogenous antigens into a host is through the skin or mucosa. Thus, delivery methods and routes which target the skin (e.g., for cutaneous and subcutaneous conditions) or mucosa (e.g., for respiratory, ocular, lingual or genital conditions) will be especially useful. Those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, means for drug delivery into skin and mucosa. For review, however, exemplary methods and routes of drug delivery useful in the invention are briefly discussed below.

Intranasal administration means are particularly useful in addressing respiratory inflammation, particularly inflammation mediated by antigens transmitted from the nasal passages into the trachea or broncheoli. Such means include inhalation of aerosol suspensions or insufflation of the polynucleotide compositions of the invention. Nebulizer devices suitable for delivery of polynucleotide compositions to the nasal mucosa, trachea and bronchioli are well-known in the art and will therefore not be described in detail here. For general review in regard to intranasal drug delivery, those of ordinary skill in the art may wish to consult Chien, *Novel Drug Delivery Systems*, Ch. 5 (Marcel Dekker, 1992).

Dermal routes of administration, as well as subcutaneous injections, are useful in addressing allergic reactions and inflammation in the skin. Examples of means for delivering drugs to the skin are topical application of a suitable pharmaceutical preparation, transdermal transmission, injection and epidermal administration.

For transdermal transmission, absorption promoters or iontophoresis are suitable

methods. For review regarding such methods, those of ordinary skill in the art may wish to consult Chien, *supra* at Ch. 7. Iontophoretic transmission may be accomplished using commercially available "patches" which deliver their product continuously via electric pulses through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

An exemplary patch product for use in this method is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or to dose periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the LECTRO PATCH product; those instructions are incorporated herein by this reference.

Epidermal administration essentially involves mechanically or chemically irritating the outermost layer of the epidermis sufficiently to provoke an immune response to the irritant. An exemplary device for use in epidermal administration employs a multiplicity of very narrow diameter, short tynes which can be used to scratch ISS-PN/IMM coated onto the tynes into the skin. The device included in the MONO-VACC old tuberculin test manufactured by Pasteur Merieux of Lyon, France is suitable for use in epidermal administration of ISS-PN/IMM. Use of the device is according to the manufacturer's written instructions included with the device product; these instructions regarding use and administration are incorporated herein by this reference to illustrate conventional use of the device. Similar devices which may also be used in this embodiment are those which are currently used to perform allergy tests.

Opthalmic administration (e.g., for treatment of allergic conjunctivitis) involves invasive or topical application of a pharmaceutical preparation to the eye. Eye drops,

topical cremes and injectable liquids are all examples of suitable mileaus for delivering drugs to the eye.

Systemic administration involves invasive or systemically absorbed topical administration of pharamaceutical preparations. Topical applications as well as intravenous and intramuscular injections are examples of common means for systemic administration of drugs.

2. Dosing parameters

A particular advantage of the ISS-PN/IMM of the invention is their capacity to exert immunomodulatory activity even at relatively minute dosages. Although the dosage used will vary depending on the clinical goals to be achieved, a suitable dosage range is one which provides up to about 1-1000 μg of ISS-PN/IMM/ml of carrier in a single dosage. Alternatively, a target dosage of ISS-PN/IMM can be considered to be about 1-10 μM in a sample of host blood drawn within the first 24-48 hours after administration of ISS-PN/IMM. Based on current studies, ISS-PN/IMM are believed to have little or no toxicity at these dosage levels.

In this respect, it should be noted that the anti-inflammatory and immunotherapeutic activity of ISS-PN/IMM in the invention is essentially dose-dependent. Therefore, to increase ISS-PN/IMM potency by a magnitude of two, each single dose is doubled in concentration. Clinically, it may be advisable to administer the ISS-PN/IMM in a low dosage (e.g., about 1 μg/ml to about 50 μg/ml), then increase the dosage as needed to achieve the desired therapeutic goal.

In view of the teaching provided by this disclosure, those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, suitable parameters for administration of ISS-PN/IMM according to the invention.

3. ISS-PN/IMM compositions

ISS-PN/IMM will be prepared in a pharmaceutically acceptable composition for delivery to a host. Pharmaceutically acceptable carriers preferred for use with the ISS-PN/IMM of the invention may include sterile aqueous of non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/ aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. A composition of ISS-PN/IMM may also be lyophilized using means well known in the art, for subsequent reconstitution and use according to the invention.

Absorption promoters, detergents and chemical irritants (e.g., keritinolytic agents) can enhance transmission of an ISS-PN/IMM composition into a target tissue. For reference concerning general principles regarding absorption promoters and detergents which have been used with success in mucosal delivery of organic and peptide-based drugs, see Chien, *Novel Drug Delivery Systems*, Ch. 4 (Marcel Dekker, 1992).

Examples of suitable nasal absorption promoters in particular are set forth at Chien, *supra* at Ch. 5, Tables 2 and 3; milder agents are preferred. Suitable agents for use in the method of this invention for mucosal/nasal delivery are also described in Chang, *et al.*, *Nasal Drug Delivery*, "Treatise on Controlled Drug Delivery", Ch. 9 and Table 3-4B thereof, (Marcel Dekker, 1992). Suitable agents which are known to enhance absorption of drugs through skin are described in Sloan, Use of Solubility Parameters from Regular Solution Theory to Describe Partitioning-Driven Processes, Ch. 5,

"Prodrugs: Topical and Ocular Drug Delivery" (Marcel Dekker, 1992), and at places elsewhere in the text. All of these references are incorporated herein for the sole purpose of illustrating the level of knowledge and skill in the art concerning drug delivery techniques.

A colloidal dispersion system may be used for targeted delivery of the ISS-PN/IMM to specific tissue. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome.

Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al., Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes encoding the antisense polynucleotides at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al., Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be

used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various well known linking groups can be used for joining the lipid chains to the targeting ligand (see, e.g., Yanagawa, *et al.*, *Nuc.Acids Symp.Ser.*, 19:189 (1988); Grabarek, *et al.*, *Anal.Biochem.*, 185:131 (1990); Staros, *et al.*, *Anal.Biochem.*, 156:220 (1986) and Boujrad, *et al.*, *Proc.Natl.Acad.Sci.USA*, 90:5728 (1993), the disclosures of which are incorporated

herein by reference solely to illustrate the standard level of knowledge in the art concerning conjugation of PNs to lipids). Targeted delivery of ISS-PN/IMM can also be achieved by conjugation of the ISS-PN/IMM to a the surface of viral and non-viral recombinant expression vectors, to an antigen or other ligand, to a monoclonal antibody or to any molecule which has the desired binding specificity.

Co-administration of a peptide drug with an ISS-PN/IMM according to the invention may also be achieved by incorporating the ISS-PN/IMM in *cis* or in *trans* into a recombinant expression vector (plasmid, cosmid, virus or retrovirus) which codes for any therapeutically beneficial protein deliverable by a recombinant expression vector. If incorporation of an ISS-PN/IMM into an expression vector for use in practicing the invention is desired, such incorporation may be accomplished using conventional techniques which do not require detailed explanation to one of ordinary skill in the art. For review, however, those of ordinary skill may wish to consult Ausubel, *Current Protocols in Molecular Biology*, *supra*.

D. *Screening for Active ISS-PN/IMM*

Confirmation that a particular compound has the properties of an ISS-PN/IMM useful in the invention can be obtained by evaluating whether the ISS-PN/IMM affects cytokine secretion and IgG antibody isotype production as described in Section A.I, above. Details of *in vitro* techniques useful in making such an evaluation are given in the Examples; those of ordinary skill in the art will also know of, or can readily ascertain, other methods for measuring cytokine secretion and antibody production along the parameters taught herein.

E. *Kits for Use in Practicing the Methods of the Invention*

For use in the methods described above, kits are also provided by the invention. Such kits may include any or all of the following: ISS-PN/IMM (conjugated or

unconjugated); a pharmaceutically acceptable carrier (may be pre-mixed with the ISS-PN/IMM) or suspension base for reconstituting lyophilized ISS-PN/IMM; additional medicaments; a sterile vial for each ISS-PN/IMM and additional medicament, or a single vial for mixtures thereof; device(s) for use in delivering ISS-PN/IMM to a host; assay reagents for detecting indicia that the anti-inflammatory and/or immunostimulatory effects sought have been achieved in treated animals and a suitable assay device.

Examples illustrating the practice of the invention are set forth below. The examples are for purposes of reference only and should not be construed to limit the invention, which is defined by the appended claims. All abbreviations and terms used in the examples have their expected and ordinary meaning unless otherwise specified.

EXAMPLE I

SELECTIVE INDUCTION OF A Th1 RESPONSE IN A HOST AFTER ADMINISTRATION OF AN ISS-PN/IMM

In mice, IgG 2A antibodies are serological markers for a Th1 type immune response, whereas IgG 1 antibodies are indicative of a Th2 type immune response. Th2 responses include the allergy-associated IgE antibody class; soluble protein antigens tend to stimulate relatively strong Th2 responses. In contrast, Th1 responses are induced by antigen binding to macrophages and dendritic cells.

To determine which response, if any, would be produced by mice who received ISS-PN/IMM according to the invention, eight groups of Balb/c mice were immunized with 10μg β-galactosidase protein (conjugated to avidin; Sigma, St. Louis, MO) to produce a model allergic phenotype. As set forth in the Table below, some of the mice received antigen alone, some received an antigen-ISS-PN conjugate or a conjugate using a mutant, non-stimulatory PN as a conjugate for the antigen, and others received the antigen in an unconjugated mixture with an ISS-PN. Naive mice are shown for reference:

Mouse Group	ISS-PN/IMM Treatment
1	None (β-gal antigen vaccinated)
2	DY1018-βgal conjugate (ISS-PN/IMM)
3	DY1019-βgal conjugate (PN/IMM)
4	DY1018 mixed with βgal (unconjugated)
5	βgal in adjuvant (alum)
6	plasmid DNA (ISS-ODN present but not expressible with antigen)
7	naive mice (no antigen priming)

DY1018 has the nucleotide sequence:

5'-TGACTGTGAACGTTCGAGATGA-3' with a phosphothioate backbone

and DY1019 has the nucleotide sequence:

5'-TGACTGTGAAGGTTGGAGATGA-3' with a phosphothioate backbone.

At 2 week intervals, any IgG 2a and IgG 1 to β-galactosidase present in the serum of each mouse were measured by enzyme-linked immunoabsorbent assay (using antibodies specific for the IgG 1 and IgG 2A subclasses) on microtiter plates coated with the enzyme.

As shown in FIGURE 1, only the mice who received the ISS-PN/IMM produced high titers of IgG 2A antibodies, which increased in number over a period of 8 weeks. As

shown in FIGURE 2, immunization of the mice with the antigen itself or with the PN/IMM induced production of relatively high titers of IgG 1 antibodies. The data shown in the FIGURES comprise averages of the values obtained from each group of mice.

To evaluate the effect of treatment of a host before and after a secondary antigen challenge, 3 groups of Balb/c mice were immunized with 10μg of antigen E (AgE) in alum to produce a model allergic phenotype and challenged again with the antigen, ISS-PN/IMM or mutant (nonstimulatory) PN/IMM at 5 weeks post-priming. An ELISA for IgG1 and IgG2a antibodies was performed as described 4 weeks after priming (one week before secondary antigen challenge) and again at 7 weeks (2 weeks after secondary challenge).

Again, the mice who received the ISS-PN/IMM mounted a strong Th1 type response to the antigen (IMM) as compared to the antigen-immunized and mutant PN/IMM immunized mice (FIGURE 3), while the reverse was true of a Th2 type response in the same mice (FIGURE 4).

These data indicate that a selective Th1 response is induced by administration of an ISS-PN/IMM according to the invention to both an antigen-primed (pre-antigen challenge) and an antigen-challenged host.

EXAMPLE II

SUPPRESSION OF IgE ANTIBODY RESPONSE TO ANTIGEN BY IMMUNIZATION WITH ISS-PN/IMM

To demonstrate the IgE suppression achieved through stimulation of a Th1 type cellular immune response in preference to a Th2 type cellular immune response, five

to eight week old Balb/c mice were immunized with AgE as described in the previous Example.

IgE anti-Age were detected using a solid phase radioimmunoassay (RAST) in a 96 well polyvinyl plate (a radioisotopic modification of the ELISA procedure described in Coligan, *"Current Protocols In Immunology"*, Unit 7.12.4, Vol. 1, Wiley & Sons, 1994), except that purified polyclonal goat antibodies specific for mouse ϵ chains were used in lieu of antibodies specific for human Fab. To detect anti-AgE IgE, the plates were coated with AgE (10μg/ml). The lowest IgE concentration measurable by the assay employed was 0.4ng of IgE/ml.

Measuring specifically the anti-antigen response by each group of mice, as shown in FIGURE 5, anti-AgE IgE levels in the ISS-PN/IMM immunized mice were consistently low both before and after boosting, while the protein and mutant ISS-PN/IMM injected mice developed high levels of anti-AgE after antigen challenge.

These data show that the ISS-PN/IMM immunized mice developed an antigen specific Th1 response (suppressing the Th2 IgE response) to the antigen.

EXAMPLE III

INFγ LEVELS IN MICE AFTER DELIVERY OF ISS-PN/IMM

BALB/c mice were immunized with βgal as described in Example I then sacrificed 24 hrs later. Splenocytes were harvested from each mouse.

96 well microtiter plates were coated with anti-CD3 antibody (Pharmingen, La Jolla, CA) at a concentration of 1μg/ml of saline. The anti-CD3 antibody stimulates T cells by delivering a chemical signal which mimicks the effects of binding to the T cell receptor (TCR) complex. The plates were washed and splenocytes added to each well

(4x105/well) in a medium of RPMI 1640 with 10% fetal calf serum. Supernatants were obtained at days 1, 2 and 3.

Th1 cytokine (INFγ) levels were assayed with an anti-INFγ murine antibody assay (*see*, e.g., Coligan, *"Current Protocols in Immunology"*, Unit 6.9.5., Vol. 1, Wiley & Sons, 1994). Relatively low levels of INF-γ would be expected in mice with a Th2 phenotype, while relatively high levels of INF-γ would be expected in mice with a Th1 phenotype.

As shown in FIGURE 5, levels of Th1 stimulated IFN-γ secretion were greatly increased in the ISS-PN/IMM treated mice, but substantially reduced in each other set of mice (as compared to the control), indicating development of a Th2-type phenotype in the latter mice and a Th1 phenotype in the ISS-PN/IMM treated mice.

EXAMPLE IV

BOOSTING OF CTL RESPONSES BY ISS-PN/IMM

A mixture of lymphoytes was obtained and contacted with βgal antigen alone or as part of the constructs and mixtures described in Example I. As shown in FIGURE 6, CTL production in response to ISS-PN/IMM was consistently higher than the response to antigen delivered in other forms; even twice as high than in animals treated with an unconjugated mixture of ISS-PN and IMM antigen.

In the experiment, the higher values for the mice treated with M-ISS-PN/IMM after antigen challenge as compared to the conventionally immunized mice is most likely owing to the antigen carrier properties of DY1019.

Thus, longer-term immunity mediated by cellular immune responses is benefitted by treatment according to the invention.

The invention claimed is:

CLAIMS

1. An immunomodulatory composition comprising an immunomodulatory molecule, which molecule comprises an antigen, conjugated to an polynucleotide that contains at least one immunostimulatory nucleotide sequence (ISS).

2. The composition of claim 1, wherein the antigen is selected from the group consisting of proteins, glycoproteins, polysaccharides and gangliosides.

3. The composition of claim 2, wherein the ISS comprises a nucleotide sequence selected from the group CpG, p(GC) and p(IC).

4. The composition of claim 2, wherein the ISS comprises a CG containing oligonucleotide.

5. The composition of claim 4, wherein the ISS further comprises a pG nucleotide sequence.

6. The composition of claim 4, wherein the CG containing oligonucleotide has the sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

7. The composition of claim 3, wherein the CpG, p(GC) or p(IC) containing nucleotide sequence is a palindromic double-stranded or non-palindromic single-stranded oligonucleotide.

8. The composition of claim 6, wherein the oligonucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

9. The composition of claim 6, wherein the oligonucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

10. The composition of claim 6, wherein the oligonucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

11. The composition of claim 2, wherein the polynucleotide further comprises a linear DNA sequence.

12. The composition of claim 2, wherein the polynucleotide further comprises a circular DNA sequence.

13. The composition of claim 2, wherein the polynucleotide further comprises an RNA nucleotide sequence.

14. The composition of claim 13, wherein the RNA nucleotide sequence comprises a sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC, GACGCU, GACGCpI, GACGCpC, GACGUU, GACGpI, GACGpC, GACGUC, GACGpI, GACGpC.

15. The composition of claim 13, wherein the RNA nucleotide sequence comprises a double-stranded poly(I•C) sequence.

16. The composition of claim 13, wherein the RNA nucleotide sequence comprises a sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC.

17. The composition of claim 13, wherein the RNA nucleotide sequence comprises a sequence selected from the group consisting of AACGUU, AACGpI, AACGpC.

18. The composition of claim 2, wherein the polynucleotide further comprises at least one modified oligonucleotide.

19. The composition of claim 11, wherein the ISS is contained within the linear DNA sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

20. The composition of claim 11, wherein the ISS is contained within the linear DNA sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

21. The composition of claim 12, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

22. The composition of claim 12, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

23. The composition of claim 13, wherein the ISS is contained within the RNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

24. The composition of claim 13, wherein the ISS is contained with the RNA nucleotide sequence, and further wherein the ISS comprises CG containing pG nucleotide sequence.

25. The composition of claim 4, wherein the CG containing nucleotide sequence further comprises a modified oligonucleotide.

26. The composition of claim 6, wherein the 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3' nucleotide sequence further comprises a modified oligonucleotide.

27. An immunomodulatory composition comprising an immunomodulatory molecule, which molecule comprises an antigen and an immunostimulatory peptide, conjugated to an polynucleotide that contains at least one ISS.

28. The composition of claim 27, wherein the polynucleotide is DNA or RNA.

29. The composition of claim 27, wherein the immunostimulatory peptide is selected from the group consisting of co-stimulatory molecules, cytokines, chemokines, targeting protein ligands, and trans-activating factors.

30. The composition of claim 27, wherein the ISS comprises a DNA or RNA nucleotide sequence selected from the group CG, p(GC) and p(IC).

31. The composition of claim 27, wherein the ISS comprises a CG containing oligonucleotide.

32. The composition of claim 31, wherein the ISS further comprises a pG nucleotide sequence.

33. The composition of claim 31, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

34. The composition of claim **31**, wherein the CG containing nucleotide sequence is a palindromic double-stranded or non-palindromic single-stranded oligonucleotide.

35. The composition of claim **33**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

36. The composition of claim **33**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

37. The composition of claim **33**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

38. The composition of claim **29**, wherein the polynucleotide further comprises a linear DNA nucleotide sequence.

39. The composition of claim **29**, wherein the polynucleotide further comprises a circular DNA nucleotide sequence.

40. The composition of claim **29**, wherein the polynucleotide portion further comprises an RNA nucleotide sequence.

41. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a nucleotide sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC, GACGCU, GACGCpI, GACGCpC, GACGUU, GACGpI, GACGpC, GACGUC, GACGpI, GACGpC.

42. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a double-stranded poly(I•C) nucleotide sequence.

43. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a nucleotide sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC.

44. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a nucleotide sequence selected from the group consisting of AACGUU, AACGpI, AACGpC.

45. The composition of claim **29**, wherein the polynucleotide portion further comprises at least one modified oligonucleotide.

46. The composition of claim **38**, wherein the ISS is contained within the linear DNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

47. The composition of claim **38**, wherein the ISS is contained within the linear DNA nucleotide sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

48. The composition of claim **39**, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

49. The composition of claim **39**, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

50. The composition of claim **40**, wherein the ISS is contained within the RNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

51. The composition of claim **40**, wherein the ISS is contained with the RNA nucleotide sequence, and further wherein the ISS comprises CG containing pG nucleotide sequence.

52. The composition of claim **31**, wherein the CG containing nucleotide sequence further comprises a modified oligonucleotide.

53. The composition of claim **33**, wherein the 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3' nucleotide sequence further comprises a modified oligonucleotide.

54. A method of modulating an immune response comprising the administration of an immunomodulatory composition comprising an immunomodulatory molecule, which molecule comprises an antigen, conjugated to an polynucleotide that contains at least one ISS.

55. The method of claim **54**, wherein the route of administration is a dermal route.

56. The method of claim **54**, wherein the route of administration is low-frequency ultrasonic delivery.

57. The method of claim **54**, wherein the antigen is selected from the group consisting of proteins, glycoproteins, polysaccharides and gangliosides.

58. The method of claim **57**, wherein the ISS comprises a DNA or RNA nucleotide sequence selected from the group CG, p(GC) and p(IC).

59. The method of claim **57**, wherein the ISS comprises a CG containing oligonucleotide.

60. The method of claim **59**, wherein the ISS further comprises a pG nucleotide sequence.

61. The method of claim **59**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

62. The method of claim **59**, wherein the CG containing nucleotide sequence is a palindromic or non-palindromic oligonucleotide nucleotide sequence.

63. The method of claim **59**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

64. The method of claim **59**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

65. The method of claim **59**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

66. The method of claim **54**, wherein the immune response modulation comprises the induction of a Th1 response.

67. The method of claim **66**, wherein the antigen molecule is selected from the group consisting of proteins, glycoproteins and polysaccharides.

68. The method of claim **67**, wherein the ISS comprises a DNA or RNA nucleotide sequence selected from the group CG, p(GC) and p(IC).

69. The method of claim **67**, wherein the ISS comprises a CG containing oligonucleotide.

70. The method of claim **69**, wherein the ISS further comprises a pG nucleotide sequence.

71. The method of claim **69**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

72. The method of claim **69**, wherein the CG containing nucleotide sequence is a double-stranded palindromic or single-stranded non-palindromic oligonucleotide nucleotide sequence.

73. The method of claim **69**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

74. The method of claim **69**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

75. The method of claim **69**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

76. A method of modulating an immune response comprising the administration of an immunomodulatory composition comprising an immunomodulatory molecule, which molecule is comprised of an antigen and an immunostimulatory peptide, conjugated to an polynucleotide that contains at least one ISS.

77. The method of claim **76**, wherein the route of administration is a dermal route.

78. The method of claim **76**, wherein the route of administration is low-frequency ultrasonic delivery.

79. The method of claim **76**, wherein the immunostimulatory peptide is selected from the group consisting of co-stimulatory molecules, cytokines, chemokines, targeting protein ligands, and trans-activating factors.

80. The method of claim **79**, wherein the ISS comprises a nucleotide sequence selected from the group CG, p(GC) and p(IC).

81. The method of claim **79**, wherein the ISS comprises a CG containing oligonucleotide.

82. The method of claim **81**, wherein the ISS further comprises a pG nucleotide sequence.

83. The method of claim **81**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

84. The method of claim **81**, wherein the CG containing nucleotide sequence is a double-stranded palindromic or single-stranded non-palindromic oligonucleotide nucleotide sequence.

85. The method of claim **81**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

86. The method of claim **81**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

87. The method of claim **81**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

88. The method of claim **76**, wherein the immune response modulation comprises the induction of a Th1 response.

89. The method of claim **88**, wherein the antigen is selected from the group consisting of proteins, glycoproteins and polysaccharides.

90. The method of claim **89**, wherein the ISS comprises a nucleotide sequence selected from the group CG, p(GC) and p(IC).

91. The method of claim **89**, wherein the ISS comprises a CG containing oligonucleotide.

92. The method of claim **91**, wherein the ISS further comprises a pG nucleotide sequence.

93. The method of claim 91, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

94. The method of claim 91, wherein the CG containing nucleotide sequence is a double-stranded palindromic or single-stranded non-palindromic oligonucleotide nucleotide sequence.

95. The method of claim 91, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

96. The method of claim 91, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

97. The method of claim 91, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

98. A method for introducing a soluble antigen into the Class I MHC processing pathway of the mammalian immune system to elicit a CTL response to the antigen comprising administering a polynucleotide conjugated to an immunomodulatory molecule, which molecule comprises the antigen, to a mammalian host.

99. The method of claim 98 wherein the polynucleotide includes at least one ISS.

100. The method of claim 98 wherein the polynucleotide is free of ISS.

101. The method of claim **98**, wherein the antigen is selected from the group consisting of proteins, glycoproteins and polysaccharides.

102. The method of claim **98**, wherein the ISS comprises a nucleotide sequence selected from the group CG, p(GC) and p(IC).

103. The method of claim **98**, wherein the ISS comprises a CG containing oligonucleotide.

104. The method of claim **103**, wherein the ISS further comprises a pG nucleotide sequence.

105. The method of claim **103**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

106. The method of claim **103**, wherein the CG containing nucleotide sequence is a double-stranded palindromic or single-stranded non-palindromic oligonucleotide nucleotide sequence.

107. The method of claim **102**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

108. The method of claim **102**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

109. The method of claim **102**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

110. The method of claim **98** wherein the polynucleotide comprises a GpG oligonucleotide.

111. The method of claim **110**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, AGGGTC, GAGGTC, GGGGTC, AAGGCC, AGGGCC, GAGGCC, GGGGCC, AAGGCT, AGGGCT, GAGGCT, and GGGGCT.

112. The composition of claim **110**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, and AGGGTC.

113. The composition of claim **110**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, and GAGGTT.

114. A composition for introducing a soluble antigen into the Class I MHC processing pathway of the mammalian immune system to elicit a CTL response to the antigen comprising a polynucleotide conjugated to an immunomodulatory molecule, which molecule comprises the antigen.

115. The composition of claim **114**, wherein the antigen is selected from the group consisting of proteins, glycoproteins and polysaccharides.

116. The composition of claim **114** wherein the polynucleotide comprises a GpG oligonucleotide.

117. The composition of claim **116**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, AGGGTC, GAGGTC, GGGGTC, AAGGCC, AGGGCC, GAGGCC, GGGGCC, AAGGCT, AGGGCT, GAGGCT, and GGGGCT.

118. The composition of claim **116**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, and AGGGTC.

119. The composition of claim **116**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, and GAGGTT.

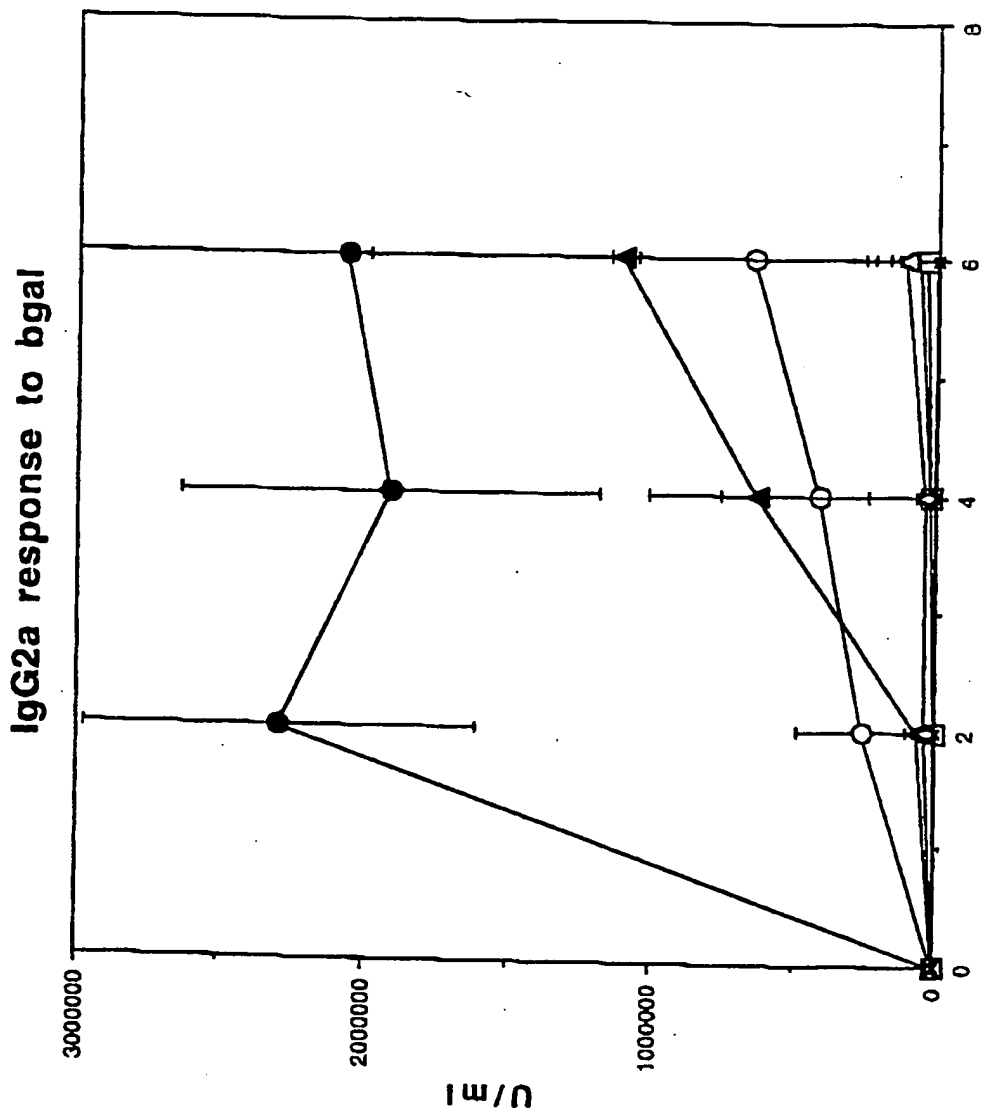
IgG2a response to bgal
U/ml
3000000
2000000
1000000
0
0
2
4
6
8
weeks
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive
mean +/- SD

FIGURE 1

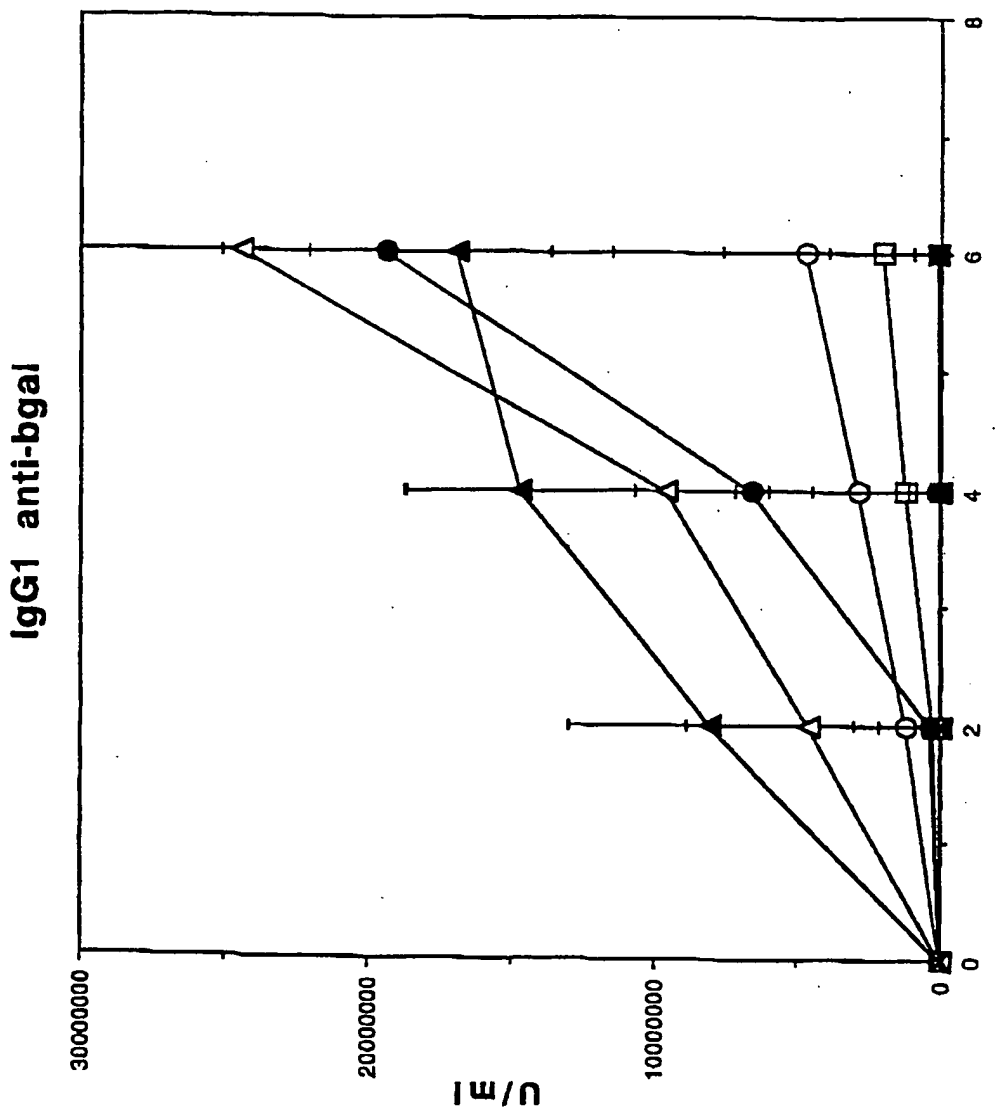
IgG1 anti-bgal
U/ml
30000000
20000000
10000000
0
0
2
4
6
8
weeks
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive
mean +/- SD

FIGURE 2

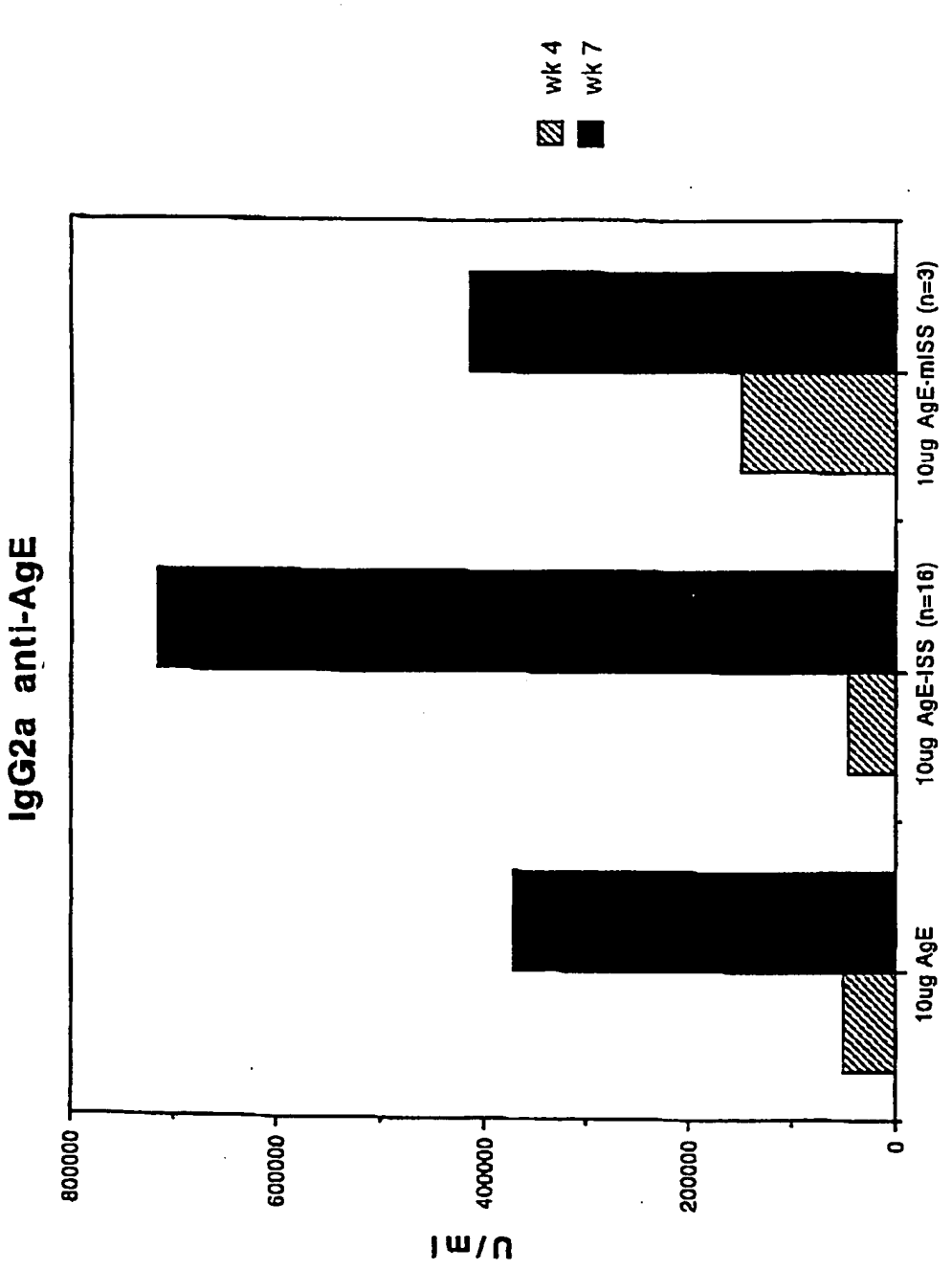
IgG2a anti-AgE
U/ml
800000
600000
400000
200000
0
wk 4
wk 7
10ug AgE
10ug AgE-ISS (n=16)
10ug AgE-mISS (n=3)

FIGURE 3

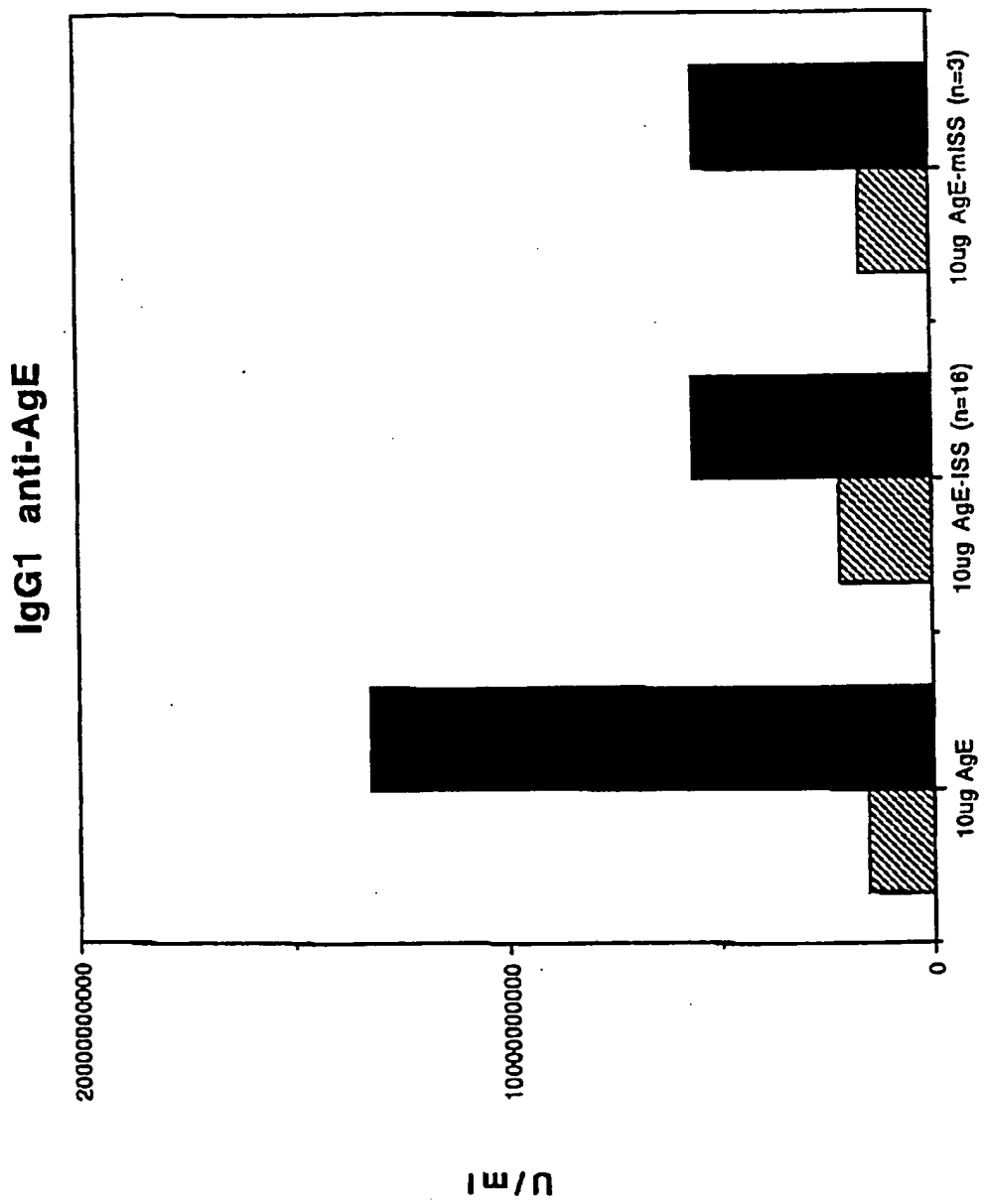
wk 4
wk 7
IgG1 anti-AgE
U/ml
20000000000
10000000000
0
10ug AgE
10ug AgE-ISS (n=16)
10ug AgE-mISS (n=3)

FIGURE 4

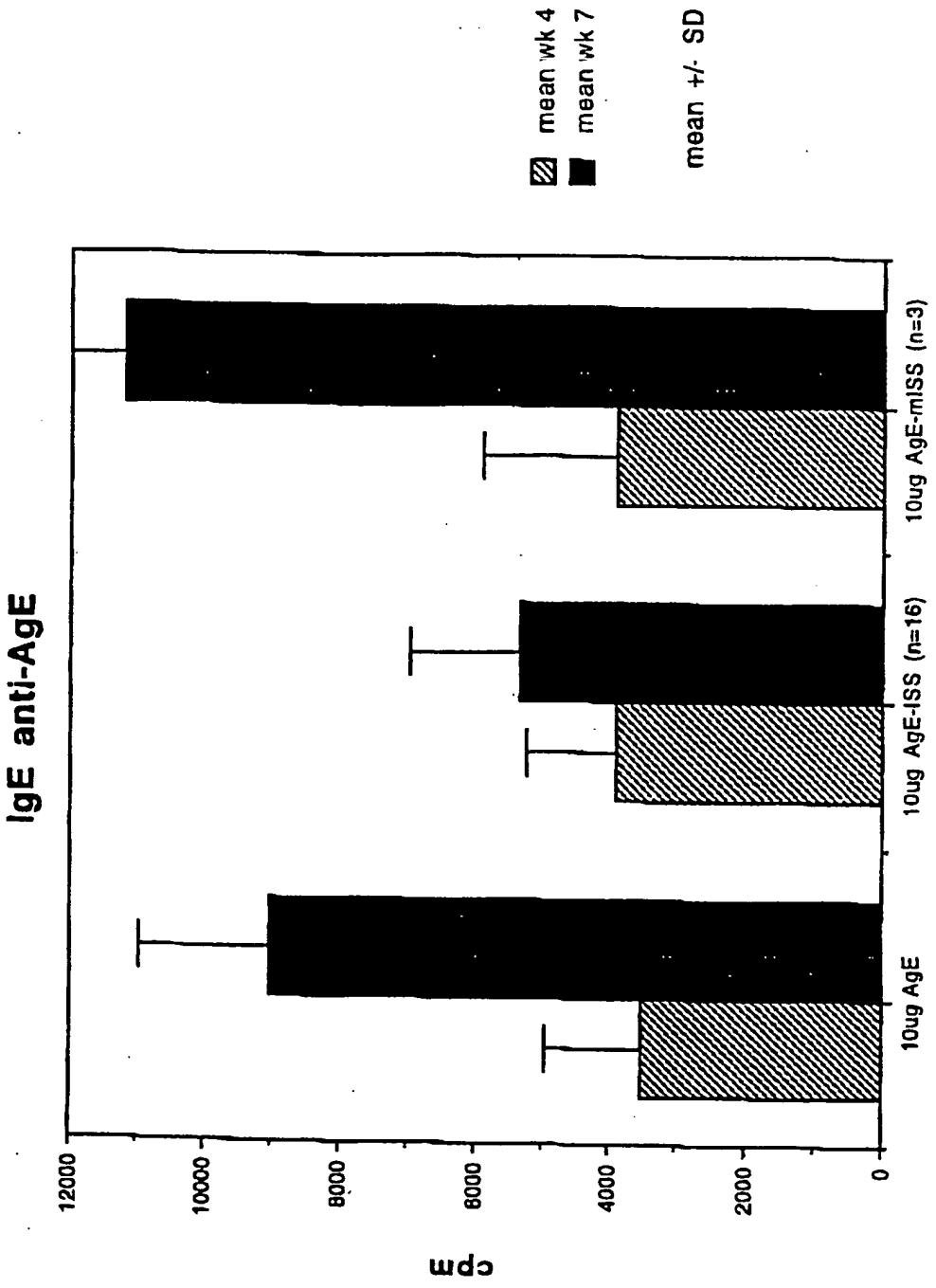
mean wk 4
mean wk 7
mean +/- SD
IgE anti-AgE
cpm
12000
10000
8000
8000
4000
2000
0
10ug AgE
10ug AgE-ISS (n=16)
10ug AgE-mISS (n=3)

FIGURE 5

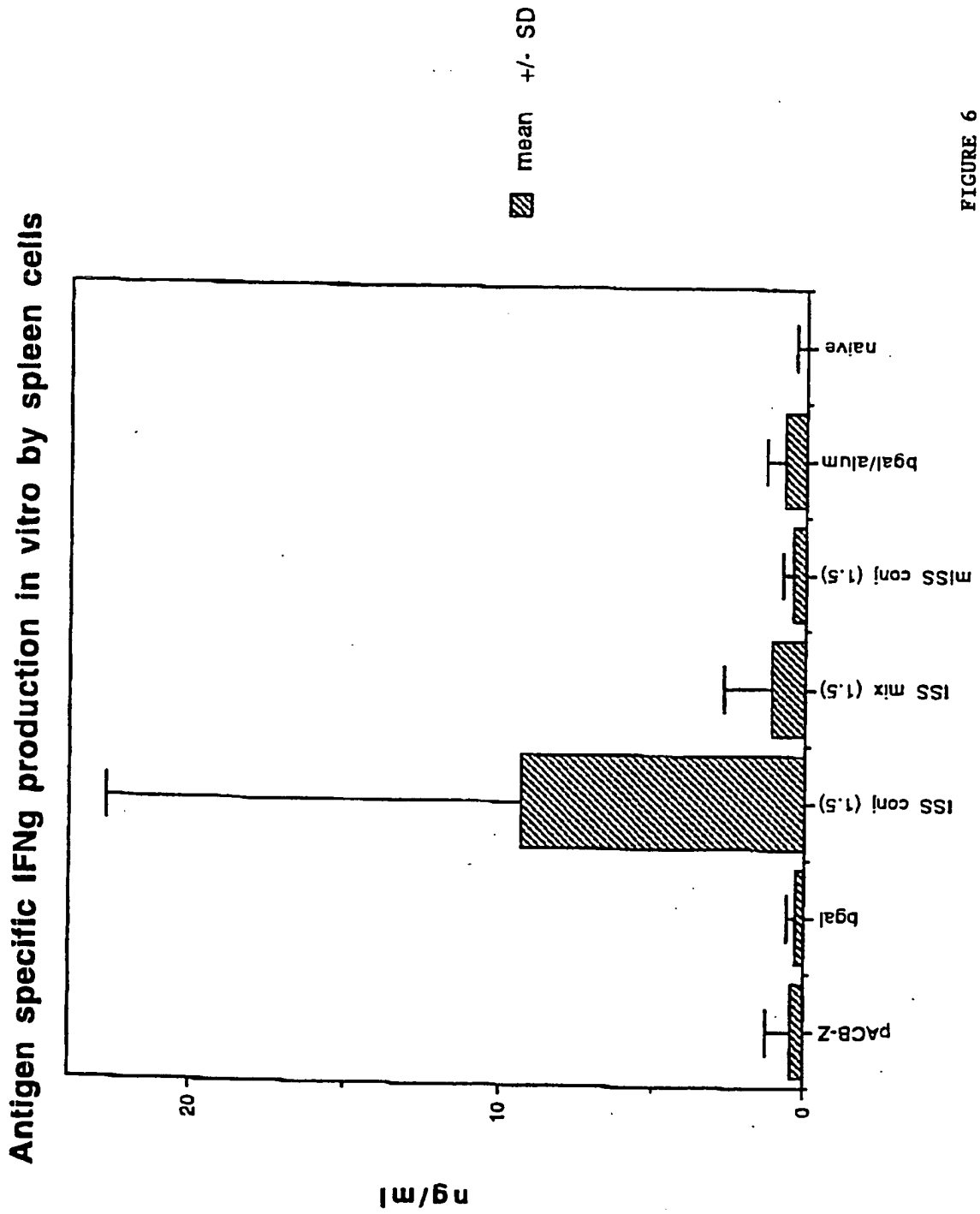
Antigen specific IFNg production in vitro by spleen cells
mean +/- SD
ng/ml
0
10
20
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive

FIGURE 6

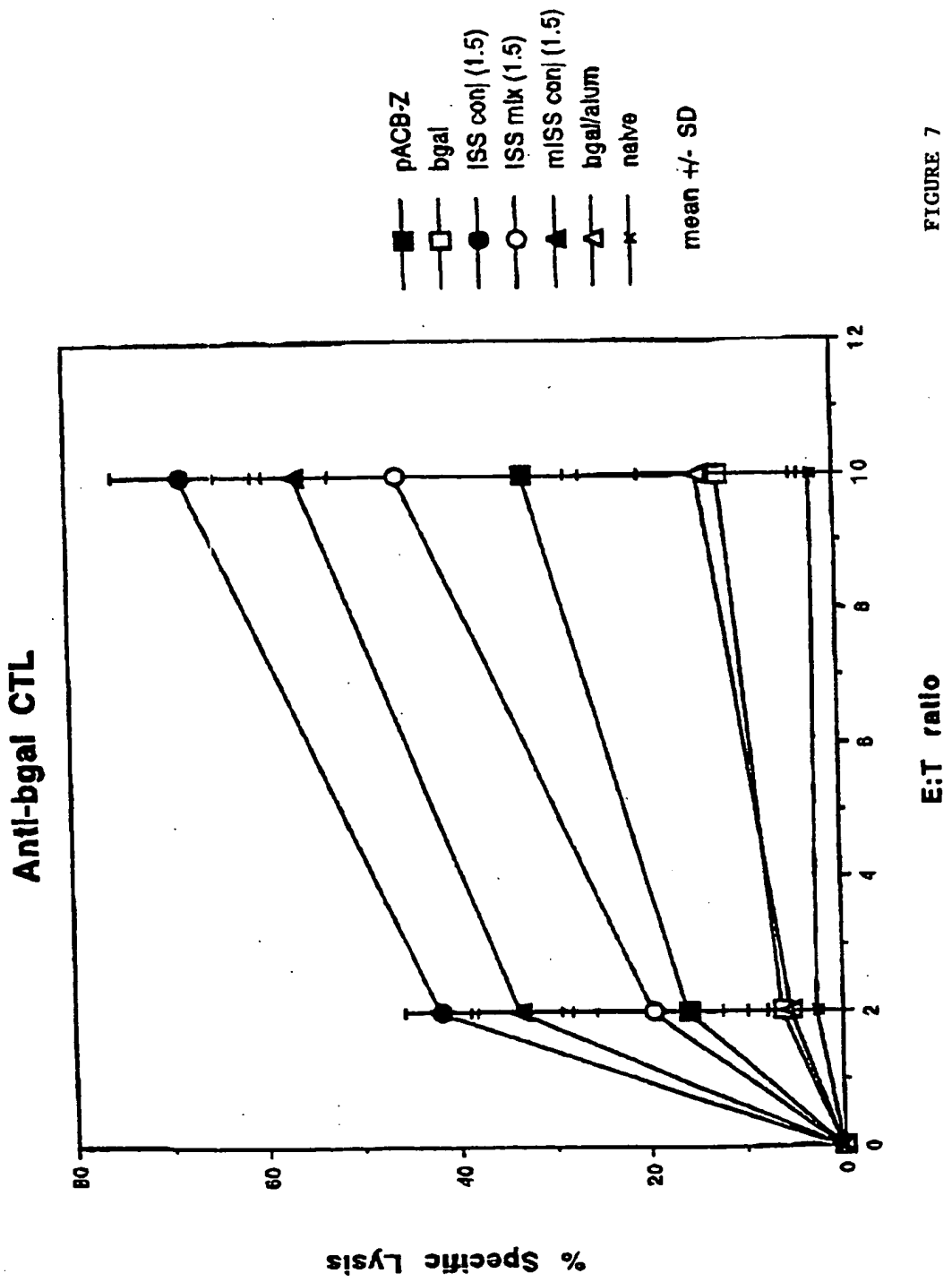
Anti-bgal CTL
% Specific Lysis
E:T ratio
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive
mean +/- SD
0
20
40
60
80
0
2
4
6
8
10
12

FIGURE 7

INTERNATIONAL SEARCH REPORT

Interm ıal Application No
PCT/US 97/19004

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 A61K39/385 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 02555 A (UNIV IOWA RES FOUND) 1 February 1996 see page 7, line 5 - page 8, line 6 see page 11, line 10 - line 20; table 1 see page 21, line 18 - line 21	1,54
Y	---	2-53, 55-119
Y	IVAN M. ROIT: "ENCYCLOPEDIA OF IMMUNOLOGY" 1992 , ACADEMIC PRESS , LONDON XP002058362 see page 28 - page 30 see page 30, left column, first paragraph	2-53, 55-119

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1998	25. 03. 1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fernandez y Branas,F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RAZ E. ET AL: "Potential role of immunostimulatory DNA sequences (ISS) in genetic immunization and autoimmunity" ARTHRITIS & RHEUMATISM, vol. 39, no. 9, September 1996, page 615 XP002058356 see the whole document ---	1-119
A	SATO Y. ET AL: "Immunostimulatory DNA sequences necessary for effective intradermal gene immunization" SCIENCE, vol. 273, July 1996, LANCASTER, PA US, XP002058357 see the whole document ---	1-119
A	ARTHUR M. KRIEG ET AL: "CpG motifs in bacterial DNA trigger direct B-cell activation" NATURE, vol. 374, 1995, LONDON GB, pages 546-549, XP002058358 see the whole document ---	1-119
A	BALLAS, Z.K. ET AL: "Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA" JOURNAL OF IMMUNOLOGY, vol. 157, September 1996, BALTIMORE US, pages 1840-1845, XP002058359 see the whole document ---	1-119
A	RAZ E. ET AL: "Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, May 1996, WASHINGTON US, pages 5141-5145, XP002058360 see the whole document ---	1-119
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A	WO 95 26204 A (ISIS PHARMACEUTICALS INC) 5 October 1995 see the whole document ---	1-119

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/19004

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3 906 092 A (HILLEMAN MAURICE R ET AL) 16 September 1975 see the whole document ---	1-119
A	US 3 725 545 A (MAES R) 3 April 1973 see the whole document ---	1-119
A	GB 1 234 718 A (MERCK) 9 June 1971 see the whole document -----	1-119

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/19004

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 112-113 (partially)

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 112-113 refer to the compositions of claim 110; however, claim 110 is a method claim. This is obscure. Hence, claims 112-113 have been understood as method claims.

Remark : Although claims 54-113
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the alleged effects of the composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern[illegible]al Application No
PCT/US 97/19004

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(54) Title: GENE EXPRESSION VECTORS WHICH GENERATE AN ANTIGEN SPECIFIC IMMUNE RESPONSE AND METHODS OF USING THE SAME

(57) Abstract

The invention consists of recombinant gene expression vectors and vaccines useful in immunization of a host against an antigen and methods for use of such vectors and vaccines. In particular, the recombinant gene expression vectors of the invention are plasmids, cosmids or viruses which include non-coding, palindromic regions of single or double-stranded DNA or RNA polynucleotides which include at least one cytosine-guanine dinucleotide motif in each palindrome. These polynucleotide regions of each expression vector are immunostimulatory and serve as adjuvants to vaccination protocols against target antigens. Most preferably, the recombinant gene expression vectors of the invention are naked; i.e., non-viral vectors not associated with a delivery vehicle such as a liposome. The invention also includes live viral vaccines wherein the viruses include immunostimulatory polynucleotides of the invention. According to a preferred method of the invention, a target protein antigen is administered through its expression by a recombinant gene expression vector which contains the non-coding, immunostimulatory polynucleotides of the invention. In the most preferred embodiment of the method of the invention, the recombinant gene expression vector is administered to tissues of the host which contain a relatively high concentration of antigen presenting cells (e.g., skin or mucosa) compared to other host tissues.

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GENE EXPRESSION VECTORS WHICH GENERATE AN ANTIGEN SPECIFIC IMMUNE RESPONSE AND METHODS OF USING THE SAME

Statement of Federally Sponsored Research

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Reference to Related Patent Applications

This is a continuation-in-part of U.S. Patent Application Serial No. 08/446,691, filed June 7, 1995, which is in turn a continuation-in-part of U.S. Patent Application Serial No. 08/112,440, filed August 26, 1993.

Field of the Invention

The invention relates to methods and reagents for immunizing a host against an antigen. Specifically, the invention relates to recombinant expression vectors for use as an adjuvant for vaccination of a host against an antigen and methods for using such vectors.

Background of the Invention

Immunization of a host against an antigen has traditionally been accomplished by repeatedly vaccinating the host with an immunogenic form of the target antigen. An emerging area of vaccine design involves the use of cytokines to direct and boost immune responses to a target antigen (which may lower the total dose of tolerizing antigen required to induce protection).

For example, the IL-12 cytokine is believed to encourage proliferation of $CD4^+$ TH1 cells (active in cell-mediated immunity) and cytotoxic T lymphocytes (CTLs) in preference to TH2 cells (active in humoral immunity). IL-12 has also been shown to substantially augment proliferation and differentiation of lymphocytes, including cytotoxic T lymphocytes. There is evidence that IL-12 plays a critical role in confering immune protection against intracellular antigens (see, e.g., Scott, *J.Immunol.*, 147:3149 (1991) [protective effect against *L.major* in mice in the presence of IL-12 lost when source of IL-12 eliminated]). However, administration of purified cytokines to a host risks toxicity, particularly at dosages sufficient to stimulate the host immune system. The same risk is posed by administration of target antigen to the host in a conventional vaccination scheme.

For these reasons, gene transfer (for introduction of a protein antigen and/or cytokine into a host by administration of a gene which encodes the antigen and/or cytokine of interest) is an intriguing alternative to traditional, antigen-based immunization protocols. However, the viral vectors commonly used for *in situ* gene expression may integrate endogenous genetic material into the host's genome and present potential health risks associated with damage to the genetic material in host cells.

Recently, "naked" gene expression vectors (e.g., plasmids for expression of a target polypeptide) have been shown to express encoded polypeptides *in vivo*. One of the earliest steps in this field was taken in 1984 at the NIH. Seeger, *et al.* reported data which indicated that intrahepatic injection of naked, cloned plasmid DNA for squirrel hepatitis into

squirrels produced both viral infection and the formation of antiviral antibodies in the squirrels (Seeger, *et al.*, *Proc.Nat'l.Acad.Sci USA*, 81:5849-5852, 1984). Several years later, Felgner, *et al.*, reported that they obtained expression of viral protein from plasmids injected into the skeletal muscle tissue of mice (Felgner, *et al.*, *Science*, 247:1465, 1990; see also, PCT application WO 90/11092).

More recently, research concerning potential therapeutic uses for naked gene expression vectors has focused on enhancing gene expression through use of different promotors, delivery vehicles and routes of administration (see, e.g., Stribling, *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:11277-11281, 1992 [expression following aerosol delivery of a gene occurred with use of a liposomal delivery system]; and, Tang, *et al.*, *Nature*, 356:152-154, 1992 [injection with a vaccine "gun" of an hGH plasmid coupled to colloidal gold beads]).

However, use of muscle as a route for gene vaccine administration has certain drawbacks. For example, researchers working with the University of Ottawa recently observed that "[s]triated muscle is the only tissue found to be capable of taking up and expressing reporter genes that are transferred in the form of plasmid DNA...but our findings indicate that fibers damaged by the injection procedure do not take up and express plasmid DNA." (Davis, *et al.*, *Human Gene Therapy*, 4:151-159, 1993).

The production of humoral immune responses to the expression products of naked gene vectors in tissues other than muscle has sparked interest in the use of the vectors as vehicles for vaccines and to deliver immunostimulatory cytokines to target cells (e.g., in recent human trials, IL-2 and IL-4

were delivered by retroviral vectors and *ex vivo* transformed cells). However, an obstacle to the use of naked gene expression vectors for vaccination has been the relatively rarity of cellular immune responses to expressed antigen.

In general, a cellular immune response to antigen (particularly through expansion of the cytotoxic T cell population) can be expected to be necessary to long-term protection against the antigen. However, any somatic cell that expresses antigen must first release the antigen into the extracellular space for uptake by antigen presenting cells before a class I restricted cytotoxic T cell response can to the antigen can be induced (see, e.g., Huang, *et al.*, *Science*, 264:961-965, 1994). Thus, it appears that enhancement of gene expression without stimulation of antigen presenting cell activity and induction of a cellular immune response will be insufficient to allow successful use of naked gene expression vectors in vaccination protocols.

Summary of the Invention

In one aspect, the invention comprises recombinant expression vectors for use in naked gene immunization ("naked gene expression vectors"). The naked gene expression vectors of the invention include immunostimulatory polynucleotides which elicit a vigorous cell-mediated immune response. The invention also includes naked gene expression vectors for use in manipulating cellular immune responses toward the TH1 compartment.

As used with respect to the invention, the term "naked gene expression vector" refers to plasmids or cosmids which include at least one non-coding, immunostimulatory

polynucleotide region, preferably also encode a peptide of interest (e.g., antigens and cytokines) and are not associated with a delivery vehicle (e.g., liposomes, colloidal particles and the like). One of the principal advantages touted for non-viral vectors has been the lack of immune responses stimulated by the vector itself. However, the inventors have discovered that vector-mediated stimulation of the host immune system is a desirable goal, and may be necessary, to permit use of naked gene expression vectors as efficient vaccination vehicles.

In particular, the design of the inventive gene expression vectors exploits the discovery that enhancement of antigen expression by recombinant expression vectors is not sufficient to provoke a protective immune response against the expressed antigen. Specifically, the relatively high expression levels achieved from those non-viral vectors commonly tested for use in gene immunization (which lack the immunostimulatory polynucleotides of the invention) may provoke humoral immune responses of varying intensity, but rarely produce the cell-mediated immune responses necessary for long-term protection against antigen.

To the latter end, the naked gene expression vectors of the invention include immunologically active regions of nucleic acids which are believed to selectively stimulate *in vivo* transcription of interferonα (IFNα) by antigen presenting cells (APCs), which in turn stimulates production of IL-12 and proliferation of cytotoxic T lymphocytes (CTLs). According to the method of the invention, antigen uptake by APCs is augmented and the host's cell-mediated response to antigen is enhanced, thus boosting the host's cellular immunity against the antigen. In this respect, the naked gene

expression vectors of the invention are particularly useful for immunizing a host against intracellular (e.g., viral) infection. The vectors are also of particular use in stimulating the TH1 compartment in preference to the TH2 compartment, thus suppressing IgE production in response to expressed antigen.

The naked gene expression vectors of the invention include one or more non-coding, immunostimulatory polynucleotides which include at least one dinucleotide sequence consisting of adjacent, unmethylated cytosine-guanine (CG) nucleotides. Immunostimulatory polynucleotides useful in the invention may be double or single-stranded DNA or RNA, but will preferably form double-stranded palindromes. Most preferably, each CG dinucleotide sequence of the immunostimulatory polynucleotides of the invention will be flanked on one side (upstream or downstream) by two or more purine nucleotides and on the other side (upstream or downstream) by two or more pyrimidine nucleotides. The naked gene expression vectors may also encode polypeptides of interest, such as antigens and cytokines.

Given the immunostimulatory properties of the immunostimulatory polynucleotides of the invention, their inclusion in other recombinant gene expression vectors and antigen-based vaccine compositions can also be expected to enhance the anti-antigen immune response of the host. Thus, another aspect of the invention includes viral recombinant gene expression vectors and non-viral recombinant gene expression vectors associated with delivery vehicles (e.g., liposomes or colloidal particles) into which immunostimulatory polynucleotides of the invention have been inserted. In addition, using the same techniques by which

immunostimulatory polynucleotides of the invention are incorporated into viral gene expression vectors, the polynucleotides may be incorporated into live viral vaccines to augment the immune response to viral antigens.

In another aspect, the invention comprises a method for immunizing a host against antigen using the naked gene expression vectors of the invention. According to a preferred method of the invention, naked gene expression vectors are introduced into tissues of the host having a relatively high concentration of antigen presenting cells (APCs) therein (e.g., skin or mucosa) as compared to other host tissues (e.g., muscle). Introduction of the naked gene expression vectors into the host may be by any suitable means, but will preferably be made by relatively non-invasive means such as chemical or mechanical irritation of the epidermis or upper cellular layers of mucosa. With co-administration of antigen or a recombinant expression vector encoding antigen, the naked gene expression vectors of the invention serve as adjuvants to enhance the immune response of a host to the antigen.

Although the invention is not to be limited by any particular mechanism of action, it is expected that introduction of the naked gene expression vectors of the invention into host APCs will encourage APC presentation of antigen by Class I processing pathways for stimulation of TH1 immune responses in preference to TH2 immune responses. A further advantage provided by the method of the invention whereby antigen is encoded by recombinant expression vectors injected into skin or mucosa is that protective immune responses may be provoked by relatively low doses of antigen (e.g., about 50μg or less). Therefore, although IL-12 is believed to suppress

cellular protein expression (and could therefore shut down antigen presentation over time), sufficient antigen expression can be achieved in the invention to provide the host immunity sought.

Brief Description of the Drawings

FIGURE 1 is a map of a pCMV-LacZ vector which contains two copies of the immunostimulatory polynucleotide palindrome AACGTT (SEQ.ID.No.1).

FIGURE 2 is a graph comparing the anti-ß-galactosidase antibody response of mice immunized intradermally with either the pCMV-LacZ naked gene expression vector, a vector which lacks an immunostimulatory polynucleotide (pKCB-LacZ), or combinations of the latter vector with KanR (KCB) and AmpR (ACB) genes or pCMV-GMCSF (encoding granulocyte-monocyte colony stimulating factor).

FIGURE 3a is a map of a pKCB-LacZ vector which lacks an immunostimulatory polynucleotide; FIGURE 3b is a map of a pKCB-LACZ vector into which one copy of the polynucleotide of SEQ.ID.No.1 was inserted (pKCB-1aaZ); FIGURE 3c is a map of a pKCB-LacZ vector into which two copies of the polynucleotide of SEQ.ID.No.1 were inserted (pKCB-2aaZ).

FIGURE 4 is a graph comparing the anti-ß-galactosidase IgG antibody response of mice immunized intradermally with respectively, the pCMV-LacZ vector, the pKCB-LacZ vector, the pKCB-1aaZ vector, the pKCB-2aaZ vector, and combinations of the pKCB-LacZ vector with KanR (KCB) and AmpR (ACB) genes.

FIGURE 5 is a graph comparing the IgG antibody response of mice to ß-galactosidase after intradermal immunization with, respectively, pCMV-LacZ or the pKCB-LacZ vector alone and in combination with pUC-19.

FIGURE 6 is a graph depicting the cellular immune (CTL) response of mice after immunization with, respectively, the pKCB-LacZ vector, the pCMV-LacZ vector or a control vector.

FIGURE 7 is a graph depicting the cellular immune (CTL) response of mice after immunization with, respectively, the pKCB-LacZ vector or the same vector in combination with the pUC-19 vector.

FIGURE 8 is a map of a pVDREtk vector suitable for insertion of immunostimulatory polynucleotides of the invention, which vector contains a ligand-inducible nuclear receptor promoter.

FIGURE 9a represents the anti-viral antigen antibody responses of mice immunized intradermally with a pCMV-NP (viral nucleoprotein) vector; FIGURE 9b compares the responses of mice injected intramuscularly with the same vector.

FIGURE 10 depicts the level of LacZ gene expression detected in Chinese hamster ovary (CHO) cells transformed with either the pCMV-LacZ or pKCB-LacZ plasmids.

FIGURE 11 is a Kaplan-Meyer survival curve for mice vaccinated against a viral antigen according to the method of the invention and for unvaccinated mice.

FIGURE 12 is a graph depicting the memory T cell responses to antigen in mice immunized with pCMV-LacZ intradermally or intramuscularly.

FIGURE 13 is a graph depicting the IgG 2a responses to antigen of mice immunized intradermally with pCMV-LacZ, intramuscularly with pCMV-LacZ or antigen.

FIGURE 14 is a graph depicting the IgG 2a responses to antigen of mice immunized with intradermally with pCMV-LacZ, intramuscularly with pCMV-LacZ or antigen.

FIGURE 15 is a graph depicting the IgG 2a response of the mice described with respect to FIGURE 13 after boosting.

FIGURE 16 is a graph depicting the IgG 1 response of the mice decribed with respect to FIGURE 13 after boosting.

FIGURE 17 is a graph of the anti-ß-galactosidase IgE antibody responses of mice immunized with the pCMV-LacZ plasmid.

FIGURE 18 depicts the anti-NP (influenza nucleoprotein) responses of mice immunized by absorption of a pCMV-NP vector or antigen through skin treated with a keratinolytic agent.

FIGURE 19 depicts the relative levels of IFN-γ production by splenocytes from mice immunized with pKCB-LacZ, pCMV-LacZ or a combination dose of pKCB-LacZ and pUC-19, then challenged with antigen.

FIGURE 20 depicts the relative levels of IL-4 production by splenocytes from mice immunized with pKCB-LacZ, pCMV-LacZ or a combination dose of pKCB-LacZ and pUC-19, then challenged with antigen.

Description of the Preferred Embodiments

I. Immunostimulatory Polynucleotides for Use in the Gene Expression Vectors of the Invention

A. Non-coding, immunostimulatory polynucleotide sequences

The non-coding immunostimulatory polynucleotides of the invention are those which stimulate CTL activity (as compared to responses to control vectors having no immunostimulatory polynucleotide of the invention) and, preferably, stimulate production of interferons (INF) by lymphocytes. In double-stranded form, such polynucleotides include at least one palindromic region (i.e., a region where the nucleotide sequence of one strand is the reverse complement of a corresponding region of the complementary strand). Each palindromic region may be as little as about 6 nucleotides in length (and of any maximum length), excluding complementary strand sequences, extrapalindromic regions, inserted restriction sites and linkers.

Further, each palindromic region of the immunostimulatory polynucleotides of the invention includes an unmethylated CG dinucleotide sequence; i.e., at least two adjacent nucleotides, where one such nucleotide is a cytosine and the other such nucleotide is a guanine. In double-stranded molecules, each CG dinucleotide sequence present in the

palindromic region of the immunostimulatory polynucleotide is palindromic; i.e., the cytosine of the CG sequence on one strand is paired with a guanine in a CG sequence on the complementary strand. In single-stranded molecules, the relative position of each CG sequence in the immunostimulatory polynucleotide is preferably 5'-CG-3' (i.e., the C is in the 5' position with respect to the G in the 3' position).

Most preferably, each CG dinucleotide sequence of each immunostimulatory polynucleotide of the invention is flanked by at least two purine nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (e.g., TC or TT) to enhance the B lymphocyte stimulatory activity of the immunostimulatory polynucleotide (see, e.g., Krieg, *et al.*, *Nature*, 374:546-549, 1995).

The immunostimulatory polynucleotides of the invention are inserted into a naked gene expression vector by techniques well known to those of ordinary skill in the art (see, e.g., Section II, *infra*). Suitable polynucleotide sequences for use as restriction sites, linkers and the like may be included in the immunostimulatory polynucleotide of the invention to be inserted into the naked gene expression vector. The immunostimulatory polynucleotides of the invention may be inserted at any location in the naked gene expression vector and will preferably be inserted at least twice so the resulting vector contains at least two palindromic regions according to the invention.

Exemplary immunostimulatory polynucleotides of the invention include:

Single-stranded DNA: AACGTT (SEQ.ID.No.1)
Double-stranded (palindromic) DNA: AACGTT (SEQ.ID.No.2)
TTGCAA

If an immunostimulatory polynucleotide such as the one described above is absent from a recombinant gene expression vector, little humoral or cellular immune response to an expressed antigen is stimulated even where levels of antigen expression is increased. For example, as shown in FIGURE 2, intradermal injection of mice with a plasmid (pCMV-LacZ; which includes two copies of the immunostimulatory polynucleotide of SEQ.ID.No.1) stimulated a substantially greater anti-antigen antibody response to the encoded reporter molecule (ß-galactosidase, or "LacZ") than was stimulated in response to intradermal injection of a plasmid containing a kanamycin resistance enzyme encoding gene (KCB) which lacks an immunostimulatory polynucleotide of the invention (see, the vector map for pKCB-LacZ in FIGURE 3a; and Example II). Immunostimularity was conferred on the pKCB-LacZ vector when one or more copies of the immunostimulatory polynucleotide of SEQ.ID.No. 1 were inserted into the vector (to form pKCB-1aaZ and pKCB-2aaZ; see, vector maps at FIGURE 3b and FIGURE 3c; data shown in FIGURE 4; and Example II), as well as after co-administration of a separate plasmid which contains two copies of the immunostimulatory polynucleotide of SEQ.ID.No.1 (pUC-19) or vector encoding granulocyte stimulating factor (pCMV-GMCSF) (FIGURE 5).

Similarly, the cellular immune responses of such mice to the pCMV-LacZ plasmid and to co-administration of pUC-19 with a

KCB plasmid were substantially greater than the response of mice injected intradermally with the pKCB-LacZ plasmid which lacks an immunostimulatory polynucleotide of the invention (see, FIGURE 6 [CTL lysis of cells transfected with pKCB-LacZ, pCMV-LacZ or control]; FIGURE 7 [CTL lysis of cells transfected with pKCB-LacZ or pKCB-LacZ with different quantities of pUC-19]; Example III; FIGURE 19 [IFN-γ production by spleen cells from mice immunized with pKCB-LacZ (low production levels), pCMV-LacZ (higher production levels) or a combination of pKCB-LacZ and pUC-19 (higher production levels)]; FIGURE 20 [IL-4 production by the same spleen cells tested for IFN-γ production in FIGURE 19]; and Example IX).

The lack of an immune response after injection of the unmodified pKCB plasmids (as compared to plasmids including the immunostimulatory polynucleotide of SEQ.ID.No.1) was particularly surprising in view of the greater levels of antigen expression obtained *in vivo* after injection of the pKCB-LacZ plasmid (as compared to the pCMV-LacZ plasmid) (see, FIGURE 10). Logically, one would expect greater expression of antigen to be reflected in the magnitude of immune response to the antigen. Yet, absent an immunostimulatory polynucleotide in a non-coding region of the expression vector, this expectation is not fulfilled *in vivo*.

Thus, contrary to present theory in the art, increasing levels of antigen expression will not necessarily enhance the immune response of an animal to the expressed antigen. In the context of the invention, it is the immunostimulatory polynucleotides of the invention, rather than just the magnitude of antigen expression, which enhance host immune responses to expressed antigen in gene immunization protocols. This activity on the part of the immunostimulatory

polynucleotides of the invention (and recombinant gene expression vectors which contain them), as well as the beneficial adjuvant effect of that activity, is unexpected given the general view in the art that DNA is a poor immunogen and that immune responses to gene expression vectors for use in gene replacement and vaccination protocols should be avoided (as compared to the desired anti-antigen response sought in the latter context).

Other exemplary immunostimulatory polynucleotides of the invention include (only one strand of each palindrome is shown):

GCGCGC (SEQ.ID.No.3) GACGTC (SEQ.ID.No.4)
AGCGCT (SEQ.ID.No.5) ATCGAT (SEQ.ID.No.6)
CGATCG (SEQ.ID.No.7) CGTACG (SEQ.ID.No.8)
CGCGCG (SEQ.ID.No.9) TCGCGA (SEQ.ID.No.10)
ACCGGT (SEQ.ID.No.11) ACGT (SEQ.ID.No.12)
GACGATCGTC (SEQ.ID.No.13) ACGATCGT (SEQ.ID.No.14)
CGACGATCGTCG (SEQ.ID.No.15)
CGACGACGATCGTCGTCG (SEQ.ID.No.16)
CAACGTTG (SEQ.ID.No.17) ACAACGTTGT (SEQ.ID.No.18)
AACAACGTTGTT (SEQ.ID.No.19)
CAACAACGTTGTTG (SEQ.ID.No.20)

Those of ordinary skill in the art will readily be able to identify other palindromic polynucleotides which (a) possess the structural characteristics of the immunostimulatory polynucleotides of the invention described above; and, (b) stimulate both humoral and cellular immune responses *in vivo* as measured by conventional detection techniques (such as those described in the Examples, *infra*). As incorporated into

naked gene expression vectors, all such polynucleotides are within the scope of this invention.

B. Preparation of immunostimulatory, antigenic and cytokine-encoding polynucleotides for insertion into the naked gene expression vectors of the invention.

As used herein, "polynucleotide" refers to a polymer of deoxyribonucleotides or ribonucleotides, in the form of a separate fragment or as a component of a larger construct. The non-coding, immunostimulatory polynucleotides of the invention may be double or single-stranded DNA or RNA inserted into recombinant expression vectors, preferably naked gene expression vectors. Such polynucleotides must also be either non-replicating or engineered by means well known in the art so as not to replicate into the host genome. The recombinant gene expression vectors of the invention may also include coding regions for expression of antigens, cytokines, T cell epitopes and other immunotherapeutically significant polypeptides.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any polynucleotide sequence from any organism, provided the appropriate probe or antibody is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligo-peptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can also be deduced from the genetic code, however, the degeneracy of the code must be taken into account.

For example, a cDNA library believed to contain a polynucleotide of interest can be screened by injecting various mRNA derived from cDNAs into oocytes, allowing sufficient time for expression of the cDNA gene products to occur, and testing for the presence of the desired cDNA expression product, for example, by using antibody specific for a peptide encoded by the polynucleotide of interest or by using probes for the repeat motifs and a tissue expression pattern characteristic of a peptide encoded by the polynucelotide of interest. Alternatively, a cDNA library can be screened indirectly for expression of peptides of interest having at least one epitope using antibodies specific for the peptides. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of cDNA of interest.

Polynucleotides for use in the invention can also be synthesized using techniques and nucleic acid synthesis equipment which are well-known in the art. For reference in this regard, see Ausubel, *et al.*, *Current Protocols in Molecular Biology*, Chs. 2 and 4 (Wiley Interscience, 1989) (genomic DNA); and, Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., New York, 1982) (cDNA). For ease of construction and use, synthesized polynucleotides and cDNAs are generally preferred for use in the recombinant gene expression vectors of the invention.

In addition to the immunostimulatory polynucleotides of the invention, the recombinant gene expression vectors of the invention may be constructed to include coding regions for peptides of therapeutic or immunostimulatory interest. For example, a mixture of polynucleotides or separately

coadministered group of polynucleotides may be of use in immunizing a host against more than one antigen and/or to further stimulate a host immune response (by, for example, including a gene operatively encoding for an immunosuppressive cytokine such as TGFß or a relevant histocompatibility protein in the recombinant gene expression vector).

The recombinant gene expression vectors of the invention may also encode peptides having more than one biological activity. For example, a polynucleotide operatively encoding for an immunostimulatory peptide may be coupled to or administered with a polynucleotide operatively encoding an antibody in such a way that both peptide and antibody will be expressed. To illustrate, administration of genes which will jointly express IL-2 and anti-gp71 may (based on results obtained with the IL-2 protein) result in localization of the antibody in tumor tissue developed in response to murine leukemia virus (MuLV) in mice (see, re results obtained with concurrent administration of IL-2/anti-gp71 mAb's, Schultz, *et al.*, *Cancer Res.*, 50:5421-5425, 1990). Further, the same vector may also encode an antigen, T cell epitope, cytokine or other polypeptides in combination.

Up to 200 polynucleotide sequences under the control of a single promoter can be expressed by an appropriate plasmid or cosmid. Such "cocktail" vectors will be of particular use in treating infections by agents of different species which cause similar symptoms. For example, there are over 100 known species of rhinoviruses which cause respiratory illnesses having similar clinical symptoms. Rather than undertaking the identification of the particular infecting species (a laborious and often inexact process), a cocktail

vaccine could be administered according to the method of the invention which is capable of stimulating an immune response to many different rhinoviruses. This approach also allows for the construction of a vaccine to various strains of HIV, using pooled isolates of envelope genes from different patients (which genes may, if necessary, then be amplified).

Known polynucleotide sequences for genes encoding such polypeptides of interest will be readily accessible to, or known by, those of ordinary skill in the art.

II. Methods for Construction of Recombiannt and Naked Gene Expression Vectors

The recombinant gene expression vectors of the invention are preferably plasmids or cosmids which include immuno-stimulatory polynucleotides of the invention, but may also be viruses or retroviruses. As discussed above, the vectors may also include gene(s) which operatively encode a peptide of interest (e.g., antigens and cytokines). Most preferably, the vectors are "naked"; i.e., not associated with a delivery vehicle (e.g., liposomes, colloidal particles and the like). For convenience, the term "plasmid" as used in this disclosure will refer to plasmids or cosmids, depending on which is appropriate to use for expression of the peptide of interest (where the choice between the two is dictated by the size of the gene encoding the peptide of interest). "Operatively encode" refers to a gene which is associated with all of the regulatory sequences required for expression of a polypeptide.

Immunostimulatory polynucleotides of the invention, as well as polynucleotides which encode antigens or cytokines, may be

conjugated to or used in association with other polynucleotides that operatively code for regulatory proteins that control the expression of these polypeptides or may contain recognition, promoter and secretion sequences. Those of ordinary skill in the art will be able to select regulatory polynucleotides and incorporate them into the recombinant gene expression vectors of the invention (if not already present therein) without undue experimentation. For example, suitable promoters for use in murine or human systems and their use are described in Ausubel, *Current Protocols in Molecular Biology*, *supra* at Ch. 1.

In general, plasmid vectors which may be used in the invention contain promoters and control sequences which are derived from species compatible with the host cell. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, *et al.*, *Gene*, 2:95, 1977). pBR322 contains genes for ampicillin (AMPR) and tetracycline resistance (the former of which includes polynucleotide fragments useful in the invention) and thus provides easy means for identifying transformed cells. However, for use in humans, the U.S. Food and Drug Administration presently prohibits use of recombinant expression vectors which may confer ampicillin resistance to the host. The pBR322 plasmid, or other microbial plasmid must also contain or be modified to contain promoters and other control elements commonly used in recombinant DNA construction.

"Control sequence(s)" or "control region" refers to specific sequences at the 5' and 3' ends of eukaryotic genes which may be involved in the control of either transcription or translation. Virtually all eukaryotic genes have an AT-rich

region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CCAAT region where X may be any nucleotide. At the 3'end of most eukaryotic genes is an AATAAA sequence which may be the signal for additional of the poly A tail to the 3'end of the transcribed mRNA.

For those vectors for use in recombinant gene expression vectors of the invention that include genes which operatively encode polypeptides of interest, preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin promoter. The early and later promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers, *et al, Nature*, 273:113, 1978). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment (Greenaway, *et al., Gene*, 18:355-360, 1982). Promoters from the host cell or related species also are useful herein.

Promoters suitable for use with prokaryotic hosts illustratively include the ß-lactamase and lactose promoter systems (Chang, *et al., Nature*, 275:615, 1978; and Goeddel, *et al., Nature*, 281:544, 1979), alkaline phosphatase, the tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.*, 8:4057, 1980) and hybrid promoters such as the tac promoter (de Boer, *et al., Proc. Natl. Acad. Sci. USA*, 80:21-25, 1983). However, other functional bacterial promoters are

suitable. Their nucleotide sequences are generally known in the art, thereby enabling a skilled worker to ligate them to a polynucleotide which encodes the peptide of interest (Siebenlist, *et al.*, *Cell*, 20:269, 1980) using linkers or adapters to supply any required restriction sites.

In addition to prokaryotes, eukaryotic microbes such as yeast cultures may also be used as source for control sequences. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used eukaryotic microorganism in this context, although a number of other strains are commonly available.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman, *et al.*, *J. Biol. Chem.*, 255:2073, 1980) or other glycolytic enzymes (Hess, *et al. J. Adv. Enzyme Reg.* 7:149, 1968; and Holland, *Biochemistry*, 17:4900, 1978) such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degraded enzymes associated with nitrogen metabolism, metallothionine, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Yeast enhancers also are advantageously used with yeast promoters.

Transcription of DNA encoding a polypeptide of interest by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins, *et al.*, *Proc.Natl.Sci.Acad.USA*, 78:993, 1981) and 3' (Lusky, *et al.*, *Mol. Cell Bio.*, 3:1108, 1983) to the transcription unit, and within an intron (Banerji, *et al.*, *Cell*, 33:729, 1983) as well as within the coding sequence itself (Osborne, *et al.*, *Mol.Cell Bio.*, 4:1293 1984). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-feto-protein and insulin). Typically, however, an enhancer from a eukaryotic cell virus will be used. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Expression vectors that contain a gene which operatively encodes a polypeptide and are intended to be introduced into eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription which may affect mRNA expression. Expression vectors may also contain a selection gene, also termed a selectable marker. Examples of suitable selectable markers for mammalian cells which are known in the art include dihydrofolate reductase (DHFR), thymidine kinase or neomycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure (i.e., by

being conferred with drug resistance or genes altering the nutrient requirements of the host cell).

Those of ordinary skill in the art will be familiar with, or may readily ascertain the identity of, viruses and retroviruses for use as recombinant expression vectors having the non-coding, immunostimulatory polynucleotides of the invention. Such artisans will also be able to construct non-viral vectors associated with delivery vehicles such as liposomes or colloidal particles without undue experimentation. Therefore, only a brief summary regarding such viral and non-viral vectors will be provided here for review.

For those embodiments of the invention which do not rely on APC recognition of polynucleotides as antigen, a colloidal dispersion system may be used for targeted delivery. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome.

Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al.*, *Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene

transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes encoding the antisense polynucleotides at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al.*, *Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-

specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

Various viral vectors that can be utilized in the invention include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated.

By inserting one or more sequences of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing the polynucleotides of interest. A separate vector can be utilized for targeted delivery of a replacement gene to the cell(s), if needed. In antisense therapy, an antisense oligonucleotide and the replacement gene may also be delivered via the same vector since the antisense oligonucleotide is specific only for target gene containing a polymorphism.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence that enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines that have deletions of the packaging signal include, but are not limited to, Ψ2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such helper cells in which the packaging signal is intact, but the structural genes are replaced by other genes

of interest, the vector can be packaged and vector virion can be produced.

It will be appreciated that the same techniques which are utilized to incorporate the immunostimulatory polynucleotides of the invention into viral gene expression vectors may be used to incorporate the sequences into live and attenuated live viruses for use as vaccines. Such modified viral vaccines can be expected to have greater immunostimulatory properties than would be found in the viral vaccine itself.

Construction of suitable vectors containing desired coding, non-coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and relegated in the form desired to construct the plasmids required.

For example, for analysis to confirm correct sequences in plasmids constructed, the ligation mixtures may be used to transform a host cell and successful transformants selected by antibiotic resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction and/or sequenced by, for example, the method of Messing, *et al.*, (*Nucleic Acids Res.*, 9:309, 1981), the method of Maxam, *et al.*, (*Methods in Enzymology*, 65:499, 1980), or other suitable methods which will be known to those skilled in the art. Size separation of cleaved fragments is performed using conventional gel electrophoresis as described, for example, by Maniatis, *et al.*, (*Molecular Cloning*, pp. 133-134, 1982).

Host cells may be transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting

transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

For purposes of monitoring expression, recombinant gene expression vectors may be modified to include genes which operatively encode known reporter polypeptides. For example, the pRSV lac-Z DNA vector described in Norton, *et al.*, *Mol. Cell. Biol.*, 5:281, (1985), may produce ß-galactosidase with protein expression. Luciferase and chloramphenicol acetyl transferase ("CAT"; see, e.g., Gorman, *et al.*, supra, re construction of a pRSV-CAT plasmid) may also be used. Convenient plasmid propogation may be obtained in *E. coli* (see, e.g., *Molecular Cloning: A Laboratory Manual*, supra.)

Two particularly preferred plasmid vectors for modification and use according to the invention are the pRSV (Rous sarcoma virus) and pCMV (cytomegalovirus) promoter vectors. Of these promoters, CMV is preferred for polynucleotides to be introduced into tissue other than muscle. This preference is based on observations that higher levels of expression are achieved in this context when the CMV promoter is employed.

A suitable protocol for isolation of the RSV promotor and its use in construction of a plasmid vector is described in Gorman, *et al.*, *Proc. Natl. Acad. Sci, USA*, 79:6777, (1982). Other preferred plasmid vectors are pREP7 and pREV which are commercially available from Invitrogen of San Diego, California. For cloning of polynucleotides, a particularly suitable plasmid for production of mRNA is the pSP64T cloning vector described by Kreig, *et al.*, *Nucleic Acids Res.*, 12:7057-7070, (1984). Any cDNA containing an initiation

codon can be introduced into this plasmid and mRNA prepared from the expressed DNA templates using conventional techniques.

Also, particularly useful vector constructs for use according to the invention are those which contain a promoter that can be switched "on" or "off" after the vector has been administered to a patient such as the ligand-inducible nuclear receptor promoters. Recombinant gene expression vectors containing such promoters are of particular use in vaccination protocols wherein the vector is introducted into the skin or mucosa, where expression can be controlled by applying the inducing ligand for absorption into the site at which the vector has been introduced.

Nuclear receptors represent a family of transcriptional enhancer factors that act by binding to specific DNA sequences found in target promoters known as response elements. Specific members of the nuclear receptor family include the primary intracellular targets for small lipid-soluble ligands, such as vitamin D_3 and retinoids, as well as steroid and thyroid hormones ("activating ligands").

Nuclear receptors activated by specific activating ligands are well suited for use as promoters in eukaryotic expression vectors since expression of genes can be regulated simply by controlling the concentration of ligand available to the receptor. For example, glucocorticoid-inducible promoters such as that of the long terminal repeat of the mouse mammary tumor virus (MMTV) have been widely used in this regard because the glucocorticoid response elements are expressed in a wide variety of cell types. One expression system which exploits glucocorticoid response elements responsive to a

wide variety of steroid hormones (e.g., dexamethasone and progesterone) is a pGREtk plasmid (containing one or more rat tyrosine amino transferase glucocorticoid response elements upstream of the herpes simplex virus thymidine kinase (tk) promoter in pBLCAT8+), transfected in HeLa cells (*see*, Mader and White, *Proc.Natl.Acad.Sci USA*, 90:5603-5607, 1993 [pGRE2tk]; and, Klein-Hitpass, *et al.*, *Cell*, 46:1053-1061, 1986 [pBLCAT8+]; the disclosures of which are incorporated herein by this reference to illustrate knowledge in the art concerning construction of suitable promoters derived from nuclear receptor response elements ["NRRE promoters"]). The pGREtk promoter (*see*, map at FIGURE 8) is particularly effective in stimulating controlled overexpression of cloned genes in eukaryotic cells (Mader and White, *supra* at 5607).

Another particularly suitable NRRE promoter for use in the invention is one which is inducible by the vitamin D_3 compound 1,25-dihydroxyvitamin D_3 and non- hypercalcemic analogs thereof (collectively, "vitamin D_3 activating ligands"). NRRE promoters inducible by vitamin D_3 activating ligands contain the vitamin D_3 receptor (VDR) response elements PurG(G/T)TCA which recognizes direct repeats separated by 3 base pairs. Vitamin D_3 response elements are found upstream of human osteocalcin and mouse osteopontin genes; transcription of these genes is activated on binding of the VDR (*see*, *e.g.*, Morrison and Eisman, *J.Bone Miner.Res.*, 6:893-899, 1991; and, Ferrara, *et al.*, *J.Biol.Chem.*, 269:2971-2981, 1994, the disclosures of which are incorporated herein by this reference to illustrate knowledge in the art of vitamin D_3 responsive inducible promoters). Recent experimental results from testing of a recombinant expression vector containing the mouse osteopontin VDR upstream of a truncated herpes simplex virus

thymidine kinase (tk) promoter suggested that 9-*cis*-retinoic acid can augment the response of VDR to 1,25-hydroxyvitamin D_3 (*see*, Carlberg, *et al.*, *Nature*, 361:657-660,1993).

Ferrara, *et al.* also described vitamin D_3 inducible promoters in recombinant expression vectors constructed using multiple copies of a strong VDR; in particular, the mouse osteopontin VDR (composed of a direct repeat of PurGTTCA motifs separated by 3 base pairs). This VDR conforms to the PurGG/TTCA consensus motifs which have previously been shown to be responsive not only to vitamin D_3, but also to thyroid hormone and/or retinoic acid. As many as three copies of the mouse VDR was inserted into pBLCAT8+; immediately upstream of the herpes simplex virus tk promoter (*see*, e.g., FIGURE 8 [map of pVDREtk]). Transfection of the resulting VDREtk vector into COS cells (producing a "VDR expression system") proved to be particularly useful in that COS cells contain the nuclear retinoid X receptor (RXR) that has been shown to act as an auxiliary factor for binding of VDR to its response element.

The VDR expression system (and functionally equivalent expression systems under the control of, for example, human osteocalcin gene promoter) is uniquely suited for use in the invention. Specifically, expression of a polynucleotide administered to a mammal according to the invention by epidermal or dermal routes (particularly the former) in a vitamin D_3 responsive expression system can be switched on by topical administration of a 1,25-dihydroxyvitamin D_3 preparation at the point of entry (and off by withdrawing the vitamin D_3 preparation and/or modulated by applying or withdrawing a source of retinoic acid to or from the point of entry). Conveniently, 1,25-dihydroxyvitamin D_3 and

nonhypercalcemic analogs thereof have been approved for use in topical preparations by the United States Food and Drug Administration for the treatment of psoriasis and are commercially available.

In vivo tests of the NRRE promoters indicate that they are inducible on systemic exposure to their corresponding response elements. Given the expected retention of polynucleotides administered dermally or epidermally at the point of entry (thus making them available for exposure to topically absorbed response elements), it can be reasonably predicted that use of NRRE promoters for expression of such polynucleotides will also permit their *in vivo* control through topical administration of appropriate NRRE promoter activating ligands (e.g., 1,25-dihydroxyvitamin D_3 transcriptional activators with a VDR expression vector for expression of the polynucleotide of interest).

Thus, use of an NRRE promoter recombinant gene expression vector for administration and expression of coding and immunostimulatory non-coding polynucleotides according to the invention permits control of expression to, for example, switch on expression when dosing is needed or switch off expression in the event of an adverse reaction to the expressed protein or peptide.

III. Pharmaceutical Preparations of Recombinant Gene Expression Vectors

Compositions of recombinant gene expression vectors may be placed into a pharmaceutically acceptable suspension, solution or emulsion. Suitable mediums include saline and may, for indications which do not rely on antigen presenting

cells for delivery of the polynucleotides into target tissue, liposomal preparations. However, as discussed further *infra* with respect to the method of the invention, it is preferred that the recombinant gene expression vectors of the invention not be conjugated to a liposome or used with any other material which may impede recognition of the vector as foreign by the host immune system.

More specifically, pharmaceutically acceptable carriers preferred for use with the naked gene expression vectors of the invention may include sterile aqueous of non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/ aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. Further, a composition of recombinant gene expression vectors may be lyophilized using means well known in the art, for subsequent reconstitution and use according to the invention.

Isotonic buffered solution is the preferred medium for maximal uptake of the naked gene expression vectors. Further, use of absorption promoters, detergents, chemical irritants or mechanical irritation means is also preferred to enhance transmission of recombinant gene expression vector

compositions through the point of entry. For reference concerning general principles regarding promoters and detergents which have been used with success in mucosal delivery of organic and peptide-based drugs, see Chien, *Novel Drug Delivery Systems*, Ch. 4 (Marcel Dekker, 1992). Specific information concerning known means and principles of nasal drug delivery are discussed in Chien, supra at Ch 5. Examples of suitable nasal absorption promoters are set forth at Ch. 5, Tables 2 and 3; milder agents are preferred. Further, known means and principles of transdermal drug delivery are also discussed in Chien, supra, at Ch. 7. Suitable agents for use in the method of this invention for mucosal/nasal delivery are also described in Chang, *et al.*, *Nasal Drug Delivery*, "Treatise on Controlled Drug Delivery", Ch. 9 and Table 3-4B thereof, (Marcel Dekker, 1992). Suitable agents which are known to enhance absorption of drugs through skin are described in Sloan, Use of Solubility Parameters from Regular Solution Theory to Describe Partitioning-Driven Processes, Ch. 5, "Prodrugs: Topical and Ocular Drug Delivery" (Marcel Dekker, 1992), and at places elsewhere in the text.

It is expected that these techniques (and others which are conventionally used to facilitate drug delivery) may be adapted to preparation of recombinant gene expression vector for use in the methods of the invention by those of ordinary skill in the art without undue experimentation. In particular, although the approaches discussed in the preceding paragraphs have not, to the inventors' knowledge, been previously used for polynucleotide delivery, it is believed that they are suitable for use to that end. For that reason, the references identified above, while not

essential to the inventive compositions and methods, are incorporated herein by this reference.

IV. Methods for *In Vivo* Use of the Naked Gene Expression Vectors of the Invention

A. Definitions

The following definitions will be of use in understanding the method of the invention.

a. "Antigen Presenting Cells", or "APC's" include known APC's such as Langerhans cells, veiled cells of afferent lymphatics, dendritic cells and interdigitating cells of lymphoid organs. The definition also includes mononuclear cells such as (1) lymphocytes and macrophages which take up and express polynucleotides according to the invention in skin and (2) mononuclear cells depicted on histological photographs contained herein. These cells are not tissue cells but are likely to be antigen presenting cells. The most important of these with respect to the present invention are those APC's which are known to be present in high numbers in epithelia and thymus dependent areas of the lymphoid tissues, including epidermis and the squamous mucosal epithelia of the buccal mucosa, vagina, cervix and esophagus (areas with "relatively high" concentrations of APC's). In addition to their definitions set forth below, therefore, "skin" and "mucosa" as used herein particularly refer to these sites of concentration of APC's. Further, "professional APCs" shall refer to cells whose primary purpose is antigen presentation; i.e., bone marrow derived cells.

b. "Detergents/Absorption Promoters" refers to chemical agents which are presently known in the art to facilitate absorption and transfection of certain small molecules, as well as peptides.

c. "Iontophoresis" refers to a known means of transdermal transmission presently used to deliver peptides continuously to a host. More specifically, it is a process that facilitates the transport of ionic species by the application of a physiologically acceptable electrical current. This process and other transdermal transmission means are described in Chien, *et al. Transdermal Drug Delivery*, "Novel Drug Delivery Systems", Ch. 7, part C, (Marcel Dekker, 1992), the relevant disclosures of which are incorporated herein by this reference for the purpose of illustrating the state of knowledge in the art concerning techniques for drug delivery.

d. "Host" refers to the recipient of the therapy to be practiced according to the invention. The host may be any vertebrate, but will preferably be a mammal. If a mammal, the host will preferably be a human, but may also be a domestic livestock or pet animal.

e. "Target tissue" refers to the tissue of the host in which expression of the polynucleotide is sought.

f. "Skin" as used herein refers to the epidermal, dermal and subcutaneous tissues of a host.

g. "Mucosa" refers to mucosal tissues of a host wherever they may be located in the body including, but not

limited to, respiratory passages (including bronchial passages, lung epithelia and nasal epithelia), genital passages (including vaginal, penile and anal mucosa), urinary passages (e.g., urethra, bladder), the mouth, eyes and vocal cords.

h. "Point of Entry" refers to the site of introduction of the polynucleotide into a host, including immediately adjacent tissue.

i. "Dermal" and "Epidermal Administration" mean routes of administration which apply the polynucleotide(s) to or through skin. Dermal routes include intradermal and subcutaneous injections as well as transdermal transmission. Epidermal routes include any means of irritating the outermost layers of skin sufficiently to provoke an immune response to the irritant. The irritant may be a mechanical or chemical (preferably topical) agent.

j. "Epithelial Administration" involves essentially the same method as chemical epidermal administration, except that the chemical irritant is applied to mucosal epithelium.

k. "IL" refers to interleukin and "IFN" refers to interferon.

l. "TH1 Response(s)" refers to a [humoral] cellular immune response that is induced preferentially by antigens that bind to and activate certain APC's; i.e., macrophages and dendritic cells.

B. Methods for introduction of the naked gene expression vectors of the invention into target tissues having substantial concentrations of antigen presenting cells: effect of use of naked gene expression vectors on the host immune response.

The method of the invention will be described with respect to the preferred embodiment for use of the naked gene expression vectors of the invention. It will be understood, however, that other recombinant expression vectors may be administered through similar routes, although use of viral expression vectors is not desirable and use of non-naked expression vectors (i.e., with a delivery vehicle) can be expected to significantly reduce the immunostimulatory activity of the immunostimulatory polynucleotides of the invention.

Although the method of the invention is not to be limited by any particular theory regarding the mechanism by which the host immune response is stimulated to provide the host with protection against antigen, the preferred method of the invention (for introduction of antigen-encoding naked gene expression vectors into APCs) is designed to selectively and efficiently boost production of TH1 (helper T cell) lymphocytes for release of IL-12 and to augment CTL activity. In this embodiment, the TH1 component of the T lymphocyte immune response is generally stimulated in preference to the antigenic stimulation of TH2 lymphocytes, which mediate production of IgE antibody.

More specifically, over the last few years it has been shown that CD4+ cells generally fall into one of two distinct subsets, the TH1 and TH2 cells. TH1 cells principally

secrete IL-2, IFNγ, IFN-α, IL-12 and TNFß (the latter two of which mediate macrophage activation and delayed type hypersensitivity) while TH2 cells principally secrete IL-4 (which stimulates production of IgE antibodies), IL-5, IL-6 and IL-10. These CD4+ subsets exert a negative influence on one another; i.e., secretion of TH1 lymphokines inhibits secretion of TH2 lymphokines and vice versa. In addition, it is believed that exposure of TH2 cells to CTLs also suppresses TH2 cell activity.

How the helper T cell subsets are differentially regulated is not completely clear. Factors believed to favor TH1 activation resemble those induced by viral infection and include intracellular pathogens, exposure to IFNγ, IFN-α, and IL-2, the presence of APCs and exposure to low doses of antigen. Factors believed to favor TH2 activation include exposure to IL-4 and IL-10, APC activity on the part of B lymphocytes and high doses of antigen. Active TH1 cells enhance cellular immunity and are therefore of particular value in responding to intracellular infections, while active TH2 cells enhance antibody production and are therefore of value in responding to extracellular infections. However, TH2 cell activity also induces IgE production through the release of IL-4, thus encouraging the formation of IgE-antigen complexes.

In mice, IgG 2A antibodies are serological markers for a TH1 type immune response, whereas IgG 1 antibodies are indicative of a TH2 type immune response. TH2 responses include the allergy-associated IgE antibody class; soluble protein antigens tend to stimulate relatively strong TH2 responses. In contrast, TH1 responses are induced by antigen binding to macrophages and dendritic cells. As shown in the data

presented in Examples VI and VII, mice injected intradermally with antigen-encoding polynucleotides preferentially produced IgG 2A antibodies indicative of TH1 responses, which in turn are indicative of the antigen being expressed intracellularly in, then presented by, APCs. In contrast, mice injected intradermally with antigen preferentially produced IgG 1 antibodies indicative of a predominant TH2 cell response.

Thus, administration of naked gene expression vectors which encode antigens (or known immunostimulatory fragments of antigens) according to the invention not only suppresses IgE antibody production, but also does so from the outset of therapy, thus avoiding the risk of anaphylaxis posed by conventional immunotherapy protocols. Specifically, administration of antigen-encoding naked gene expression vectors (particularly through dermal and epidermal routes) selectively stimulates the production of CD4+ TH1 and CD8+ lymphocytes over CD4+ TH2 lymphocytes, stimulates IL-12 and INF-α production, and stimulates INFγ secretion (which suppresses IgE antibody activity).

As reflected in the data presented in Example VI, intradermal challenge with a protein antigen (ß galactosidase) selectively induces TH2 responses in mice which, consistent with conventional immunotherapy responses, is gradually replaced by a TH1 response in antigen desensitized mice. However, as demonstrated in Example VII, IgE antibody levels produced in the protein injected mice are substantially greater during the initial phase of treatment than are produced at any stage of treatment of mice injected with a naked gene expression vector (pCMV-LacZ) that operatively encodes the same antigen and includes an immunostimulatory polynucleotide of the invention (SEQ.ID.No.1).

Further, in mice challenged with an intradermal dose of the plasmid, the TH1 cell responses greatly exceeded those of TH2 cells. Even more surprisingly, IgE and IL-4 levels in the pCMV-LacZ challenged mice are very low, while antigen-stimulated CTL levels and TH1 cell secretion of interferons are enhanced as compared to protein challenged and control mice. Moreover, the protection against IgE production afforded to the pCMV-LacZ challenged mice continues despite subsequent challenge with the plasmid or protein, even when combined with adjuvant (Examples IV, V and VII).

C. Methods for introduction of the naked gene expression vectors of the invention into target tissues having substantial concentrations of antigen presenting cells: routes of administration and dosing protocols.

The naked gene expression vectors of the invention may be used as adjuvants in conventional vaccination protocols or may be used in gene immunization protocols; i.e., where the target antigen is a protein antigen encoded by a naked gene expression vector (which may also be the vector that contains the non-coding, immunostimulatory polynucleotides of the invention). The latter approach is preferred and will be discussed in detail below with respect to dosing and administration protocols. Isolated, non-recombinant antigen will be administered according to conventional vaccination techniques.

Many infectious antigens enter the body through the skin or mucosa, where local immunity to such antigens would be of use. For this reason, as well as the relatively high

concentration of APCs present in the mammalian skin and mucosa, these tissues are the preferred target tissues of the invention.

For dermal routes of administration, the means of introduction may be by epidermal administration, subcutaneous or intradermal injection. Of these means, epidermal administration is preferred for the greater concentrations of APCs expected to be in intradermal tissue.

The means of introduction for dermal routes of administration which are most preferred, however, are those which are least invasive. Preferred among these means are transdermal transmission and epidermal administration.

For transdermal transmission, iontophoresis is a suitable method. Iontophoretic transmission may be accomplished using commercially available "patches" which deliver their product continuously through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

An exemplary patch product for use in this method is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or to dose periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the

LECTRO PATCH product; those instructions are incorporated herein by this reference.

Epidermal administration essentially involves mechanically or chemically irritating the outermost layer of the epidermis sufficiently to provoke an immune response to the irritant. Specifically, the irritation should be sufficient to attract APC's to the site of irritation. As discussed previously, it is believed that the APC's then take up and express the administered naked polynucleotide.

An exemplary mechanical irritant means employs a multiplicity of very narrow diameter, short tynes which can be used to irritate the skin and attract APC's to the site of irritation, to take up naked polynucleotides transferred from the end of the tynes. For example, the MONO-VACC old tuberculin test manufactured by Pastuer Merieux of Lyon, France contains a device suitable for introduction of naked gene expression vectors of the invention. Another suitable device for use in the invention is a tyne device manufactured for use in allergy testing by Lincoln Diagnostics of Decatur, IL. (and sold under the trademark MULTITEST®).

Such devices typically consist of a plastic container having a syringe plunger at one end and a tyne disk at the other. The tyne disk supports a multiplicity of narrow diameter tynes of a length which will just scratch the outermost layer of epidermal cells. In the present invention, each needle is coated with a pharmaceutical composition of naked gene expression vectors by immersing the tips of the tynes into an aqueous solution of the polynucleotides. For convenience, the tyne device may then be frozen so that the polynucleotides become dried onto the tines and can be

administered without having to prepare the device for use at the time of treatment.

Use of the device is according to the manufacturer's written instructions included with the device product; these instructions regarding use and administration are incorporated herein by this reference to illustrate conventional use of the device (see also, Example VII).

Another suitable approach to epidermal administration of naked polynucleotides is by use of a chemical which irritates the outermost cells of the epidermis, thus provoking a sufficient immune response to attract APC's to the area. An example is a keratinolytic agent, such as the salicylic acid used in the commercially available topical depilatory creme sold by Noxema Corporation under the trademark NAIR. This approach may also be used to achieve epithelial administration in the mucosa. The chemical irritant may also be applied in conjunction with the mechanical irritant (as, for example, would occur if the MONO-VACC type tyne were also coated with the chemical irritant). The naked gene expression vector may be suspended in a carrier which also contains the chemical irritant or coadministered therewith (see, Example VIII).

For mucosal administration, the means of introduction will vary according to the location of the point of entry. Particularly for immunization to and treatment of respiratory infections, intranasal administration means are most preferred. These means include inhalation of aerosol suspensions or insufflation of the naked gene expression vectors of the invention. Suppositories and topical preparations will also be suitable for introduction to certain

mucosa, such as genital and ocular sites. Also of particular interest with respect to vaginal delivery of naked gene expression vectors of the invention are vaginal sandwich-type rings and pessaries. Examples of these devices and their use are described in Chien, *supra* at Ch.9.

The dosage of each naked gene expression vector to be supplied according to the method of the invention will vary depending on the desired response by the host and the polynucleotide used. Generally, it is expected that up to 100-200 μg of polynucleotide can be administered in a single dosage, although as little as about 0.3 μg of polynucleotide administered through skin or mucosa can induce long lasting immune responses.

For purposes of the invention, however, it is sufficient that the naked gene expression vectors be supplied at a dosage sufficient to cause expression of the antigenic polypeptide encoded by the polynucleotide. These dosages may be modified to achieve therapeutic, subtherapeutic or immunostimulatory levels of expression. Means to confirm the presence and quantity of expressed peptides are well-known to those skilled in the art and will not, therefore, be described in detail. Certain such means are illustrated in the Examples provided below; generally, they include immunoassays (such as enzyme-linked immunosorbent assays), PCR techniques, and immunohistological analyses performed according to techniques which are well known in the art. Dosages of the administered polynucleotides can be adjusted to achieve the desired level of expression based on information provided by these detection and quantification means as well as *in vivo* clinical signs known to practitioners skilled in the clinical arts.

Preferably, naked gene expression vectors of the invention will be administered in in "low" doses (e.g., in mice, about 50μg immunostimulatory polynucleotide or less). Those of ordinary skill in the art will readily be able to determine an equivalent dosage level for use in humans. Those of ordinary skill in the art will be familiar with the course of dosing employed in vaccination and immunotherapy protocols (i.e., priming, booster and maintenance dosing), which course will be suitable for use in the method of the invention. Generally, it can be expected that doses of less than about 50μg immunostimulatory polynucleotide, and even less than about 10μg, will be suitable for priming, booster and maintenance doses in humans. Alternatively, the priming dose of antigen-encoding polynucleotide may be followed by booster and/or maintenance doses of antigen.

Examples illustrating aspects of each embodiment of the invention are provided below. They should be regarded as illustrating rather than limiting the invention, which is defined by the appended claims. Conventional abbreviations (e.g., "ml" for milliliters) are used throughout the Examples.

EXAMPLE I

EXPRESSION OF A VIRAL PROTEIN FOLLOWING INTRADERMAL INJECTION OF A NAKED GENE EXPRESSION VECTOR

To demonstrate the competence of naked gene expression vectors of the invention for expression in the dermis, the gene for influenza ribonucleoprotein (NP) was subcloned into a pCMV plasmid. NP genes from numerous strains of influenza are known in the art and are highly conserved in sequence

among various strains (see, e.g. Gorman, *et al.*, *J. Virol*, 65:3704, 1991).

Four eight week old Balb/c mice were injected three times with 15μg of pCMV-RNP suspended in 100 μl of HBSS. Injections were made intradermally at the base of the tails at two week intervals. CTLs recognize antigens presented by class I MHC molecules and play an important role in the elimination of virally infected cells. Intramuscular (i.m.) immunization by means of cDNA expression vectors should be an effective method to introduce antigen into class I MHC molecules and thus stimulate CTL responses. In this study, intradermal (i.d.) injection of a plasmid containing the influenza nucleoprotein (NP) antigen gene induced both NP-specific CTL and high titers of anti-NP antibodies. These antibodies reached a maximum 6 weeks after injection and persisted unchanged for at least 28 weeks, in the absence of local inflammation.

Plasmid DNA was purified by CsCl banding in the presence of ethidium bromide and was stored frozen in 10 mM Tris-HCL, 0.1 mM EDTA, pH 8.0. Before injection, the plasmid was precipitated in ethanol and dissolved in normal saline containing 0.1 mM EDTA.

The presence of anti-NP IgG in serum was measured by ELISA substantially as described in Viera, *et al.*, *Int. Immunl.*, 2:487, (1990). The results of this assay are shown in FIGURE 9a; all of the animals developed high titer anti-NP antibodies, which persisted for more than 20 weeks. As shown in FIGURE 9b, the intradermal injections appeared to give about four fold higher antibody titers than intramuscular injections of equivalent amounts of plasmid DNA.

The axes of FIGURES 9a and 9b represent, respectively, the ELISA titer (mean, 1 ounce) against time. Serum dilution for all graph points is 2560.

EXAMPLE II

IN VIVO ANTIBODY RESPONSES TO THE IMMUNOSTIMULATORY POLYNUCLEOTIDES OF THE INVENTION

To compare humoral immune responses to naked gene expression vectors containing the immunostimulatory polynucleotides of the invention to humoral immune responses to vectors lacking such polynucleotides, the pCMV-LacZ plasmid described in Example I (which includes two copies of the immunostimulatory polynucleotide of SEQ.ID.No.1) was modified to substitute a gene encoding an enzyme which confers kanamycin resistance (KanR). The resulting plasmid (pKCB-LacZ) lacks any of the immunostimulatory polynucleotides of the invention (see, vector maps in FIGURES 1 [pCMV-LacZ] and 3 [pKCB-LacZ]). In contrast, the AmpR containing pCMV-LacZ plasmid includes the AACGTT (SEQ.ID.No.1) palindromic sequence at two separate locations in the vector within the AmpR gene.

Four Balb/c mice per group were each injected intradermally at the base of the tail with 50μg of either the pCMV-LacZ or pKCB-LacZ plasmids. Each injection was repeated twice at one week intervals. A third group of mice was injected with pKCB-LacZ and supplementally injected with pUC-19, a plasmid which includes the AmpR gene. As a control, a fourth group of mice was injected with a non-specific bacterial DNA. For comparison of the overall immune response elicited, a fifth group was injected with a naked gene expression vector which operatively encodes GM-CSF (granulocyte-monocyte colony

stimulating factor). Anti-antigen antibody production was measured by serum ELISA after 6 weeks.

As shown in FIGURE 2, the mice injected with pCMV-LacZ produced antibodies against the expressed LacZ reporter molecule. However, no antibody formation was detected in the sera of the mice who received the pKCB-LacZ plasmid, despite the higher level of LacZ expression achieved by the vector (detected as a measure of ß-galactosidase activity in Chinese hamster ovary cells transfected separately with each vector; see, FIGURE 10). Yet anti-LacZ antibody production was restored with co-administration of pKCB-LacZ and pUC-19 (FIGURE 5), although no such response was detected after injection of the control plasmid (*id.*). The enhancing effect of the pUC-19 vector exceeded even the response to the GM-CSF encoding vector (*id.*).

To determine the effect of the immunostimulatory polynucleotides of the invention on humoral immune responses, the pKCB-LacZ plasmid was modified to include one or two copies of the AACGTT polynucleotide palindrome found in the AmpR gene (pKCB-1aaZ [1 copy] and pKCB-2aaZ [2 copies]). For comparison, groups of pKCB-LacZ and pCMV-LacZ injected mice were also injected with, respectively, KCB or CMV plasmids which lacked the LacZ reporter molecule. Antibody responses to LacZ were measured at 4 weeks after 3 weeks of immunization as described above.

As shown in FIGURE 4, virtually no antibody response to LacZ was measured in the mice injected with pKCB-LacZ or pKCB-LacZ/pKCB, while antibody responses were detected in the mice injected with pCMV-LacZ and pCMV-LacZ/pCMV. Moreover, the mice injected with the modified KCB plasmids produced

substantially greater antibody titers than even the mice injected with the pCMV plasmids, which responses increased in proportion to the number of copies of the AACGTT polynucleotide (SEQ.ID.No.1) present in the plasmid. The enhanced response as compared to the pCMV plasmids (which contain two copies of the AACGTT polynucleotide) is probably attributable to the greater levels of antigen expression achieved by the KCB vectors (see, FIGURE 10).

EXAMPLE III

IN VIVO CTL ACTIVITY IN RESPONSE TO TO THE IMMUNOSTIMULATORY POLYNUCLEOTIDES OF THE INVENTION

To determine whether the immunostimulatory polynucleotides of the invention (i.e., palindromic, CG containing sequences) stimulate cellular as well as humoral responses, the lytic activity of CTLs after immunization of mice with either pKCB-LacZ or pCMV-LacZ was tested. A control group of mice was immunized with the antigen in alum.

36 weeks after immunization (performed as described in Example II), the mice were sacrificed and splenocytes were removed for use in standard mixed lymphocyte cultures. The cultures were grown in the presence of a known synthetic ß-galactosidase peptide. The cultures were assayed for anti-LacZ CTL activity 5-6 days, measured as a function of the percent lysis of cells exposed to the antigen by pulsing versus the effector (antigen):target ratio.

As shown in FIGURE 6, as the effector:target ratio was increased, the CTL activity in cultures of cells from the pCMV-LacZ injected mice increased from about 18% to nearly 100%. In contrast, the CTL activity in cultures from the

pKCB-LacZ and control injected mice barely exceeded 20% lytic activity even when the effector:target ratio was raised to 36:1.

To determine the effect of the two copies of the immunostimulatory polynucleotide (AACGTT) of SEQ.ID.No.1 in the pCMV-LacZ plasmid, another group of pKCB-LacZ injected mice received a co-injection of either 5µg or 100µg of pUC-19. An increase in CTL activity to nearly 60% lysis was achieved in the latter group (FIGURE 7).

EXAMPLE IV

IMMUNE RESPONSE TO VIRAL CHALLENGE BY MICE INTRADERMALLY INJECTED WITH NAKED GENE EXPRESSION VECTORS CONTAINING IMMUNOSTIMULATORY POLYNUCLEOTIDES OF THE INVENTION

To test whether immunity generated by vaccination with naked gene expression vectors of the invention could protect animals from a lethal viral challenge, groups of 10 Balb/c mice were injected intradermally 3 times with 15 μg of a pCMV plasmid (pCMV-NP) which contained two copies of the immunostimulatory polynucleotide of SEQ.ID.No. 1 and the NP gene from an H1N1 strain of influenza virus (A/PR/8/34; provided by Dr. Inocent N. Mbawvike at the Baylor College of Medicine, U.S.) Control groups included uninjected animals as well as animals injected with an irrelevant plasmid (pnBL3).

Six weeks after the initial plasmid injections, the animals were challenged with a LD_{90} dose of an H3N2 influenza strain (A/HK/68); also provided by Dr. Mbawuike). Intradermally

vaccinated mice were significantly protected from the challenge (P(0.01) as compared to unvaccinated control mice; see, FIGURE 11 (a Kaplan-Meyer survival curve).

EXAMPLE V

PROLONGED IMMUNOLOGIC MEMORY AFTER INTRADERMAL ADMINISTRATION OF NAKED POLYNUCLEOTIDES INDUCED BY ANTIGEN STIMULATION OF T CELLS

To test whether the protective effect observed in the mice described in Example IV included long-term immunologic protective memory, 0.1, 1, 10 and 100 µg of naked gene expression vectors (0.5-5 ng/1 mg DNA endotoxin content) encoding the *E.coli* enzyme β-galactosidase under the control of the CMV promoter were administered to groups of 4 mice\dosage\route either intramuscularly ("IM") or intradermally ("ID"). Each plasmid included two copies of the immunostimulatory polynucleotide of SEQ.ID.No.1 (pCMV-LacZ).

As a control, another group of 4 mice\dosage received 100 µg β-galactosidase protein ("PR") intradermally. All injections were made using 50 µl normal saline as carrier. IM and ID injections were made with a 0.5 ml syringe and a 28.5 gauge needle. Antibodies were thereafter measured by enzyme-linked immunoabsorbent assay at 2 week intervals.

Total anti-β galactosidase antibodies were measured using β-galactosidase (Calbiochem, CA) as the solid phase antigen. Microtiter plates (Costar, Cambridge, MA) were coated with 5 µg of antigen dissolved in 90mM borate (pH 8.3) and 89mM NaCl (i.e., borate buffered saline; BBS) overnight at room temperature and blocked overnight with 10 mg/ml of bovine serum albumin in BBS.

Serum samples were serially diluted in BBS starting at a 1:40 dilution for the first 8 weeks, them a 1:320 dilution thereafter. These samples were added to the plates and stored overnight at room temperature. Plates were washed in BBS+0.05% polysorbate 20, then reacted with a 1:2000 dilution of alkaline phosphatase labeled goat anti-mouse IgG antibody (Jackson Immunoresearch Labs., West Grove, PA) for 1 hour at room temperature, or were reacted with a 1:2000 dilution of alkaline phosphatase labeled goat anti-mouse IgG 1 antibody (Southern Biotech of AL), or were reacted with a 1:500 dilution of alkaline phosphatase labled rat anti-mouse IgG 2A antibody (Pharmingen, of CA), under the same conditions. Plates were washed again, then a solution of 1 mg/ml of p-nitrophenol phosphate (Boehringer-Mannheim, Indianapolis, IN) in 0.05 M carbonate buffer (pH 9.8), containing 1mM $MgCl_2$ was added. Absorbance at 405 nm was read 1 hour after addition of substrate to the plates.

Lesser antibody responses were measured in the animals who had received the pCMV Lac-Z plasmids by IM injection than by ID injection (data not shown).

To assess for T cell memory, the animals were then boosted with 0.5 μg of PR at a separate site by ID injection. If these animals had developed memory T cells to control production of antibody to β-galactosidase, they would be expected to mount a more vigorous immune response after boosting with soluble protein antigen than had been demonstrated in response to the priming dose of antigen.

As shown in FIGURE 12, it is clear that the animals which had received ID injections of pCMV-LacZ plasmid had developed substantially better immunological memory than did animals

which had received either IM injections of plasmid or of PR. Further, the memory which was developed by the ID injected animals persisted for a minimum of about 12 weeks.

EXAMPLE VI

SELECTIVE INDUCTION OF A TH1 RESPONSE AFTER INTRADERMAL ADMINISTRATION OF NAKED POLYNUCLEOTIDES

In mice, IgG 2A antibodies are serological markers for a TH1 type immune response, whereas IgG 1 antibodies are indicative of a TH2 type immune response. TH2 responses include the allergy-associated IgE antibody class; soluble protein antigens tend to stimulate relatively strong TH2 responses. In contrast, TH1 responses are induced by antigen binding to macrophages and dendritic cells. TH1 responses are to be of particular importance in the treatment of allergies and AIDS.

To determine which response, if any, would be produced by mice who received naked gene expression vectors according to the invention, mice were vaccinated with the pCMV-LacZ vector described in Example V or protein as described in Example V. At 2 week intervals, any IgG 2a and IgG 1 to β-galactosidase were measured by enzyme-linked immunoabsorbent assay (using antibodies specific for the IgG 1 and IgG 2A subclasses) on microtiter plates coated with the enzyme.

As shown in FIGURE 13, only the mice who received the plasmid by ID injection produced high titers of IgG 2A antibodies. As shown in FIGURE 14, immunization of the mice with the enzyme itself ("PR") induced production of relatively high titers of IgG 1 antibodies. In the IM injected mice, low titers of both IgG 2A and IgG 1 antibodies were produced

without apparent selectivity. The data shown in the FIGURES comprise averages of the values obtained from each group of 4 mice.

To determine the stability of the antibody response over time, the same group of animals were boosted with 0.5 μg of enzyme injected intradermally. As shown in FIGURES 15 and 16 boosting of ID injection primed animals with the enzyme induced a nearly 10-fold rise in IgG 2A antibody responses (i.e., the antibody titer rose from 1:640 to 1:5120), but did not stimulate an IgG 1 response. These data indicate that the selective TH1 response induced by ID administration of naked polynucleotides is maintained in the host, despite subsequent exposure to antigen.

EXAMPLE VII

SUPPRESSION OF IgE ANTIBODY RESPONSE TO ANTIGEN BY IMMUNIZATION WITH ANTIGEN-ENCODING POLYNUCLEOTIDES

Using the experimental protocol described in Examples V and VI, five to eight week old Balb/c mice were immunized with one of two naked gene expression vectors of the invention: the pCMV-LacZ plasmid described in Example V or a control plasmid, pCMV-BL (which does not encode for any insert peptide and does not contain immunostimulatory polynucleotides). A third group of the mice received injections of antigen (β galactosidase). Plasmid DNA was purified and its endotoxin content reduced to 0.5-5ng/1mg DNA by extraction with TRITON X-114 (Sigma, St. Louis, MI). Before inoculation, pDNA was precipitated in ethanol, washed with 70% ethanol and dissolved in pyrogen free normal saline.

Immunization was by intradermal injection of plasmid DNA loaded onto separate tynes of a MONOVACC® multiple tyne device (Connaught Lab, Inc., Swiftwater, PA). Briefly, the tyne devices were prepared after extensive washing in DDW and overnight soaking in 0.5% SDS (sulfated dodecyl saline), washed again in DDW, soaked overnight in 0.1N NaOH, washed again in DDW and dried at 37°C for 8 hours. Six µl of plasmid DNA dissolved in normal saline were pipetted onto the tynes of the tyne device just prior to each inoculation described below. The total amount of pDNA loaded on the device per inoculation was 25 µg each of pCMV-LacZ and pCMV-BL. For purposes of estimating actual doses, it was assumed that less than 10% of the pDNA solution loaded onto the tyne device was actually introduced on injection of the tynes into intradermal tissue.

Each mouse was treated 3 times with 2 inoculations of each plasmid in a one week interval injected intradermally at the base of the tail. Another group of mice received a single intradermal injection in the base of the tail of 10µg of β galactosidase protein (dissolved in 50µl of normal saline) in lieu of pDNA.

Toward inducing an IgE antibody response to subsequent antigen challenge, each group of mice was injected once intraperitoneally with 0.1 ml of phosphate buffered saline (PBS) solution containing 1µg of antigen (β galactosidase; Calbiochem, San Diego, CA) and 3mg of ALUM aluminum hydroxide as adjuvant (Pierce Chemical, Rockford, IL) 14 weeks after the initial immunization. Total IgE was assayed in sera from the mice 4 times over the subsequent 4 consecutive weeks.

IgE was detected using a solid phase radioimmunoassay (RAST) in a 96 well polyvinyl plate (a radioisotopic modification of the ELISA procedure described in Coligan, *"Current Protocols In Immunology"*, Unit 7.12.4, Vol. 1, Wiley & Sons, 1994), except that purified polyclonal goat antibodies specific for mouse ε chains were used in lieu of antibodies specific for human Fab. To detect anti-LacZ IgE, the plates were coated with β galactosidase (10μg/ml). The lowest IgE concentration measurable by the assay employed was 0.4ng of IgE/ml.

Measuring specifically the anti-antigen response by each group of mice, as shown in FIGURE 17, anti-LacZ IgE levels in the plasmid injected mice were consistently low both before and after boosting (averaging about 250 CPM in RAST), while the protein injected mice developed high levels of anti-LacZ, particularly after the first antigen booster injection, when anti-LacZ levels in the mice rose to an average of about 3000 CPM. Consistent with acquisition of tolerance, anti-LacZ IgE levels in the protein injected mice declined over time, but continued to rise in the control mice who had not received any immunization to β galactosidase.

These data show that the plasmid injected mice developed an antigen specific TH1 response to the plasmid expression product, with concomitant suppression of IgE production, while tolerance was acquired in the protein injected mice only after development of substantially higher levels of total and antigen specific IgE antibodies.

EXAMPLE VIII

EPIDERMAL ADMINISTRATION OF A NAKED GENE EXPRESSION VECTOR USING A CHEMICAL AGENT TO ELICIT AN IMMUNE RESPONSE

FIGURE 18 depicts the results of an ELISA performed as described in Example I for serum levels of anti-NP IgG following epidermal administration of the pCMV-NP vector described in Example I in conjunction with the application of a chemical agent.

The plasmid was suspended in 40 μg of an isotonic normal saline solution containing approximately 150 μg of plasmid per milliliter. This solution was absorbed onto the nonadhesive pad of a BAND-AID® brand bandage (Johnson & Johnson).

A Balb/c mouse was shaved along the base of its tail and a commercially available keratinolytic agent (here, the previously described depilatory creme sold under the trademark NAIR®) was applied to the shaved skin. After several minutes, the keratinolytic agent was washed off of the skin and the plasmid-containing bandage applied thereto. As shown in FIGURE 18, the treated animal developed serum anti-NP IgG at a titer of 1:640.

EXAMPLE IX

ENHANCEMENT OF INTERFERON AND CYTOKINE (IL-4) PRODUCTION IN ANIMALS IMMUNIZD WITH IMMUNOSTIMULATORY POLYNUCLEOTIDE CONTAINING PLASMIDS

Two groups of mice were immunized with either pCMV-LacZ or pKCB-LacZ as described in Example III. A third group of mice received a combination dose of pKCB-LacZ and pUC-19 as

described in Example II. After sacrifice, splenocytes were removed and challenged *in vitro* with β-galactosidase antigen. The release of IFN-γ and IL-4 into supernatants from the antigen challenged cells was measured.

Mice immunized with pKCB-LacZ alone produced little IFN-γ and IL-4 as compared to mice immunized with pCMV-LacZ or the combination pKCB-LacZ/pUC-19 dose (see, FIGURES 19 and 20).

The invention having been fully described, other embodiments and modifications of the invention may be apparent to those of ordinary skill in the art. All such embodiments and modifications are within the scope of the invention, which is defined by the appended claims.

SUMMARY OF SEQUENCES

SEQ.ID.No.1 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.2 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.3 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.4 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.5 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.6 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.7 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.8 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.9 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.10 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.11 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.12 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.13 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.14 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.15 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.16 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.17 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.18 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.19 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQ.ID.No.20 is a non-coding, immunostimulatory polynucleotide useful in the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Carson, Dennis A.
Raz, Eyal

(ii) TITLE OF INVENTION: RECOMBINANT GENE EXPRESSION VECTORS AND METHODS FOR USE OF SAME TO ENHANCE THE IMMUNE RESPONSE OF A HOST TO AN ANTIGEN

(iii) NUMBER OF SEQUENCES: 20

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson P.C.
(B) STREET: 4225 Executive Square, Suite 1400
(C) CITY: La Jolla
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 92037

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30B

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Howells, Stacy L.
(B) REGISTRATION NUMBER: 34,842
(C) REFERENCE/DOCKET NUMBER: 07340/044001

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (619) 678-5070
(B) TELEFAX: (619) 678-5099

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AACGTT 6

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTGCAA 6

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCGCGC 6

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GACGTC 6

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGCGCT 6

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATCGAT 6

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGATCG 6

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGTACG 6

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCGCG 6

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCGCGA 6

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACCGGT 6

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACGT 4

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GACGATCGTC 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACGATCGT 8

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..12

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGACGATCGT CG 12

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGACGACGAT CGTCGTCG 18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CAACGTTG 8

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ACAACGTTGT 10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..12

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AACAACGTTG TT 12

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..14

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAACAACGTT GTTG 14

CLAIMS

1. A recombinant gene expression vector into which at least one non-coding, immunostimulatory polynucleotide has been inserted, wherein the immunostimulatory polynucleotide is comprised of at least one strand of a palindrome, wherein the palindrome includes at least one dinucleotide consisting of adjacent, unmethylated cytosine and guanine nucleotides.

2. The recombinant gene expression vector of Claim 1 wherein the vector is a plasmid or cosmid.

3. The recombinant gene expression vector of Claim 2 wherein the plasmid or cosmid is naked.

4. The recombinant gene expression vector of Claim 1 wherein the palindrome of the immunostimulatory polynucleotide is at least 6 nucleotides in length.

5. The recombinant gene expression vector of Claim 1 wherein the dinucleotide in the palindrome consists of a cytosine nucleotide in the 5' position and a guanine nucleotide in the 3' position.

6. The recombinant gene expression vector of Claim 1 wherein the dinucleotide in the palindrome consists of a cytosine nucleotide in the 3' position and a guanine nucleotide in the 5' position.

7. The recombinant gene expression vector of Claim 5 wherein at least two purine nucleotides are upstream and adjacent to the cytosine nucleotide of the dinucleotide and at least two pyrimidine nucleotides are downstream and adjacent to the guanine nucleotide.

8. The recombinant gene expression vector of Claim 5 wherein at least two pyrimidine nucleotides are upstream and adjacent to the cytosine nucleotide of the dinucleotide and at least two purine nucleotides are downstream and adjacent to the guanine nucleotide.

9. The recombinant gene expression vector of Claim 6 wherein at least two purine nucleotides are upstream and adjacent to the guanine nucleotide of the dinucleotide and at least two pyrimidine nucleotides are downstream and adjacent to the cytosine nucleotide.

10. The recombinant gene expression vector of Claim 6 wherein at least two pyrimidine nucleotides are upstream and adjacent to the guanine nucleotide of the dinucleotide and at least two purine nucleotides are downstream and adjacent to the cytosine nucleotide.

11. The recombinant gene expression vector of Claim 1 further comprising a polynucleotide which encodes a polypeptide.

12. The recombinant gene expression vector of Claim 11 wherein the polypeptide is an antigen or immunostimulatory antigen fragment.

13. The recombinant gene expression vector of Claim 11 wherein the polypeptide is a cytokine.

14. The recombinant gene expression vector of Claim 11 wherein the polypeptide is a T lymphocyte epitope.

15. The recombinant gene expression vector of Claim 11 wherein expression of the encoded polynucleotide is under the control of a nuclear receptor promoter.

16. The recombinant gene expression vector of Claim 1 wherein the immunostimulatory polynucleotide is selected from the group of polynucleotides having one strand which consists of AACGTT (SEQ.ID.No.1); GCGCGC (SEQ.ID.No.3); GACGTC (SEQ.ID.No.4); AGCGCT (SEQ.ID.No.5); ATCGAT (SEQ.ID.No.6); CGATCG (SEQ.ID.No.7); CGTACG (SEQ.ID.No.8); CGCGCG (SEQ.ID.No.9); TCGCGA (SEQ.ID.No.10); ACCGGT (SEQ.ID.No.11); ACGT (SEQ.ID.No.12); GACGATCGTC (SEQ.ID.No.13); ACGATCGT (SEQ.ID.No.14); CGACGATCGTCG (SEQ.ID.No.15); CGACGACGATCGTCGTCG (SEQ.ID.No.16); CAACGTTG (SEQ.ID.No.17); ACAACGTTGT (SEQ.ID.No.18); AACAACGTTGTT (SEQ.ID.No.19), or CAACAACGTTGTTG (SEQ.ID.No.20).

17. A recombinant gene expression vector consisting essentially of pKCB-1aaZ.

18. A recombinant gene expression vector consisting essentially of pKCB-2aaZ.

19. A method for enhancing the immune response of a host to an antigen comprising introducing a recombinant gene expression vector into a tissue of the host, wherein the recombinant gene expression vector into which at least one non-coding, immunostimulatory polynucleotide has been inserted, wherein the immunostimulatory polynucleotide is comprised of at least one strand of a palindrome, wherein the palindrome includes at least one dinucleotide consisting of adjacent, unmethylated cytosine and guanine nucleotides.

20. The method according to Claim 19 wherein the recombinant gene expression vector comprises a plasmid or cosmid.

21. The method according to Claim 20 wherein the recombinant gene expression vector is naked.

22. The method according to Claim 19 wherein the host tissue into which the recombinant gene expression vector is introduced is a tissue which has a high concentration of antigen presenting cells relative to other host tissues.

23. The method according to Claim 22 wherein the host tissue is skin or mucosa.

24. The method according to Claim 19 wherein the recombinant gene expression vector encodes at least one polypeptide.

25. The method according to Claim 24 wherein at least one of the encoded polypeptides is the antigen or an immunostimulatory fragment of the antigen.

26. The method according to Claim 24 wherein at least one of the encoded polypeptides is a cytokine.

27. The method according to Claim 24 wherein at least one of the encoded polypeptides is a T lymphocyte epitope.

28. The method according to Claim 25 wherein the antigen is expressed in the antigen presenting cells of the host tissue.

29. A method according to Claim 19 wherein the recombinant gene expression vector is coated onto the tynes of a multiple tyne device and is administered by penetrating the skin of the host with the tynes.

30. A method according to Claim 19 wherein the recombinant gene expression vector is introduced by absorption through skin or mucosa treated with a keratinolytic agent.

31. A method according to Claim 24 wherein expression of the encoded polypeptide by the recombinant gene expression vector is under the control of a nuclear receptor promoter.

32. A DNA or RNA virus into which at least one non-coding, immunostimulatory polynucleotide has been inserted, wherein the immunostimulatory polynucleotide is comprised of at least one strand of a palindrome, wherein the palindrome includes at least one dinucleotide consisting of adjacent, unmethylated cytosine and guanine nucleotides.

33. The virus of Claim 32 wherein the palindrome of the immunostimulatory polynucleotide is at least 6 nucleotides in length.

34. The virus of Claim 32 wherein the dinucleotide in the palindrome consists of a cytosine nucleotide in the 5' position and a guanine nucleotide in the 3' position.

35. The virus of Claim 32 wherein the dinucleotide in the palindrome consists of a cytosine nucleotide in the 3' position and a guanine nucleotide in the 5' position.

36. The virus of Claim 34 wherein at least two purine nucleotides are upstream and adjacent to the cytosine nucleotide of the dinucleotide and at least two pyrimidine nucleotides are downstream and adjacent to the guanine nucleotide.

37. The virus of Claim 34 wherein at least two pyrimidine nucleotides are upstream and adjacent to the cytosine nucleotide of the dinucleotide and at least two purine nucleotides are downstream and adjacent to the guanine nucleotide.

38. The virus of Claim 35 wherein at least two purine nucleotides are upstream and adjacent to the guanine nucleotide of the dinucleotide and at least two pyrimidine nucleotides are downstream and adjacent to the cytosine nucleotide.

39. The virus of Claim 35 wherein at least two pyrimidine nucleotides are upstream and adjacent to the guanine nucleotide of the dinucleotide and at least two purine nucleotides are downstream and adjacent to the cytosine nucleotide.

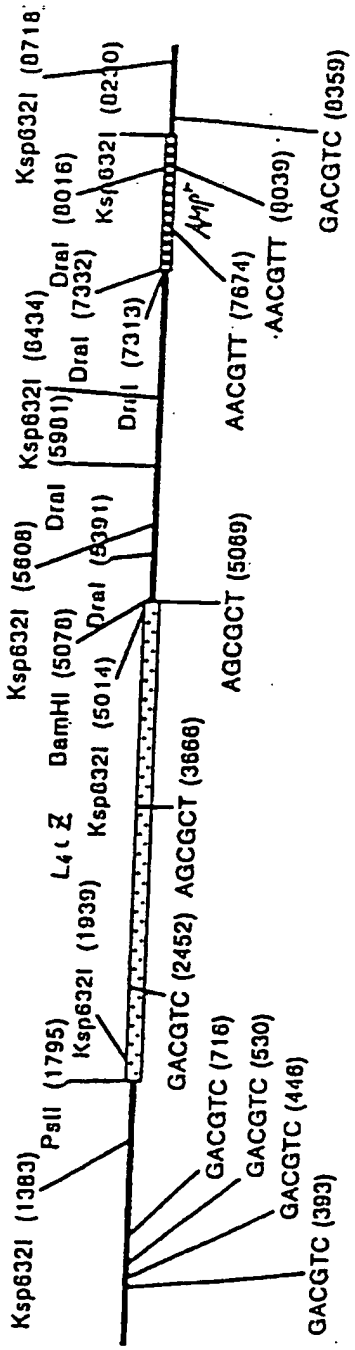

Ksp632I (0718)
Ksp632I (0210)
GACGTC (0359)
Ksp632I (0016)
Ksp632I (0039)
AAGTT
DraI (7332)
AACGTT (7674)
DraI (7313)
Ksp632I (6434)
DraI (5901)
DraI
Ksp632I (5808)
DraI (5391)
Ksp632I (5070)
DraI (5014)
AGCGCT (5089)
DamHI
Ksp632I (3866)
L4cZ
Ksp632I (1939)
GACGTC (2452) AGCGCT
Ksp632I (1795)
PsiI (1383)
GACGTC (716)
GACGTC (530)
GACGTC (446)
GACGTC (393)

FIGURE 1

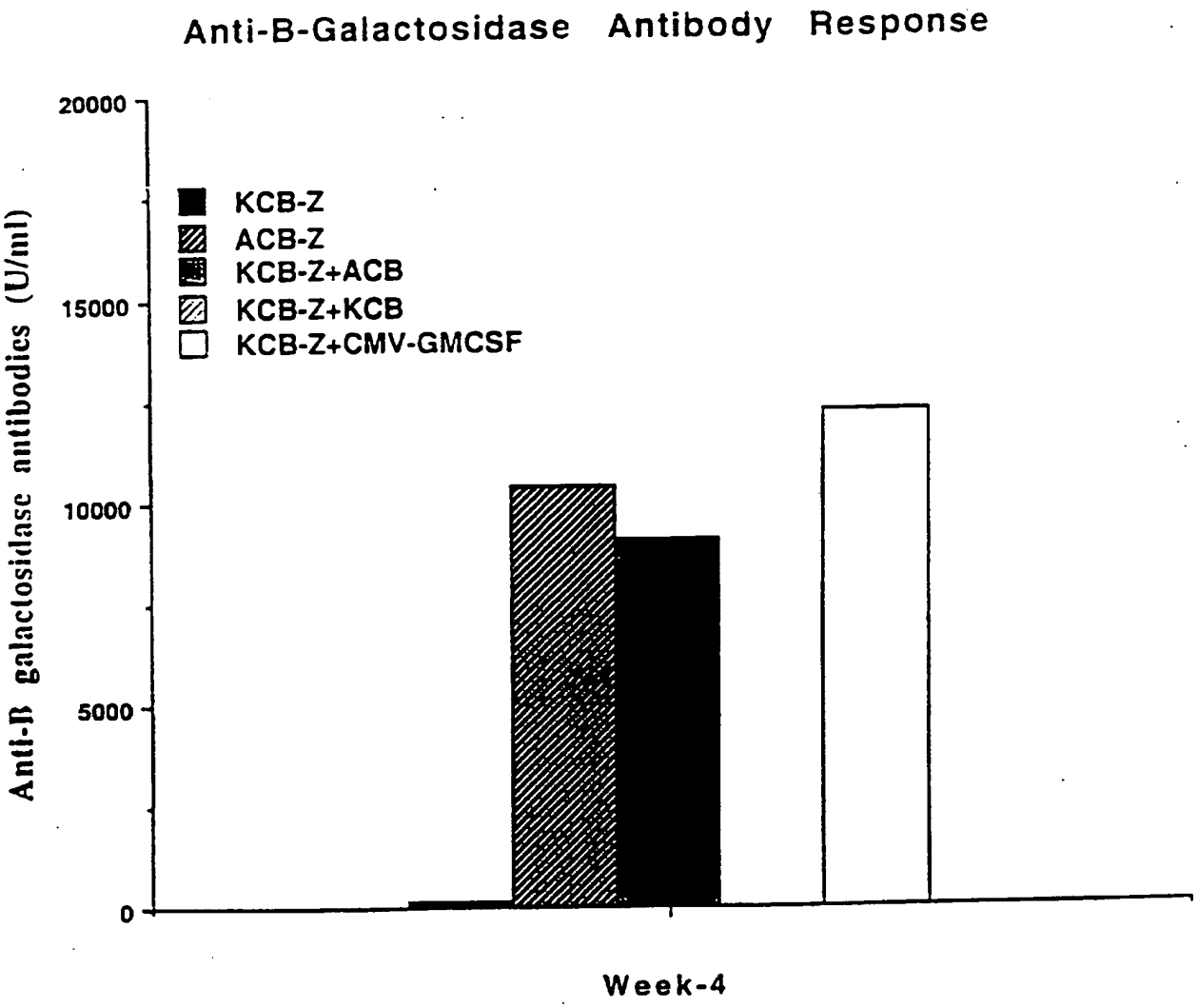

Anti-B-Galactosidase Antibody Response
20000
15000
10000
5000
0
Anti-β galactosidase antibodies (U/ml)
KCB-Z
ACB-Z
KCB-Z+ACB
KCB-Z+KCB
KCB-Z+CMV-GMCSF
Week-4

FIGURE 2

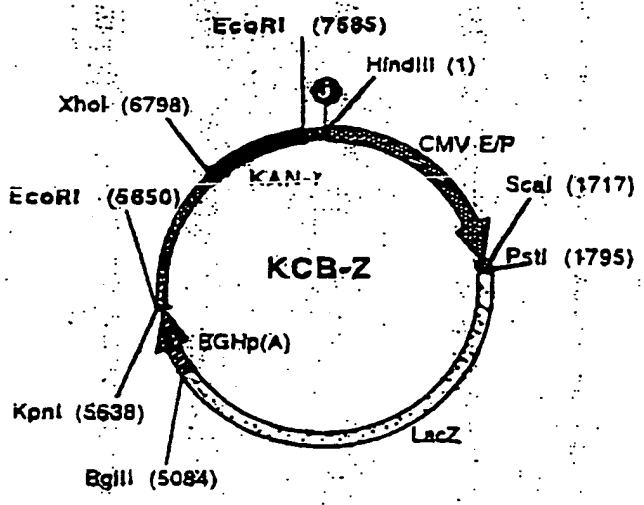


FIGURE 3a

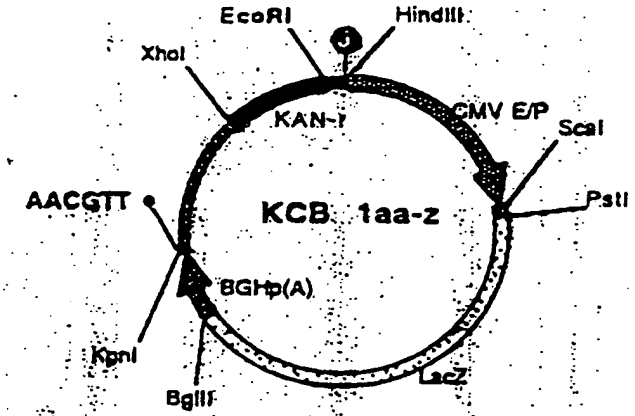


FIGURE 3b

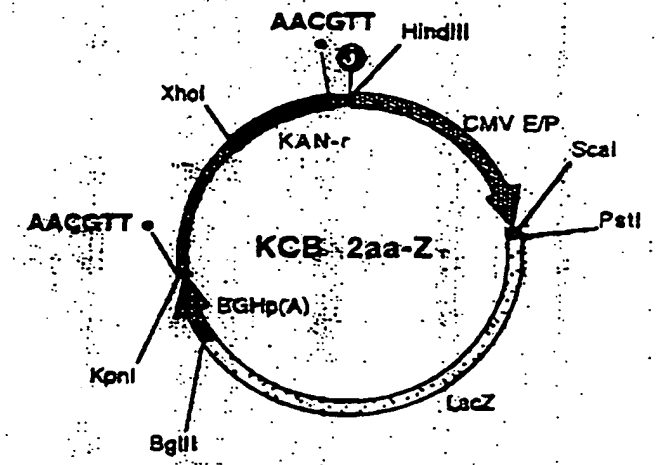


FIGURE 3c

AACGTT: AATTGAACGTTCGC
CTTGCAAGCGTTAA

FIGURES 3a-3c

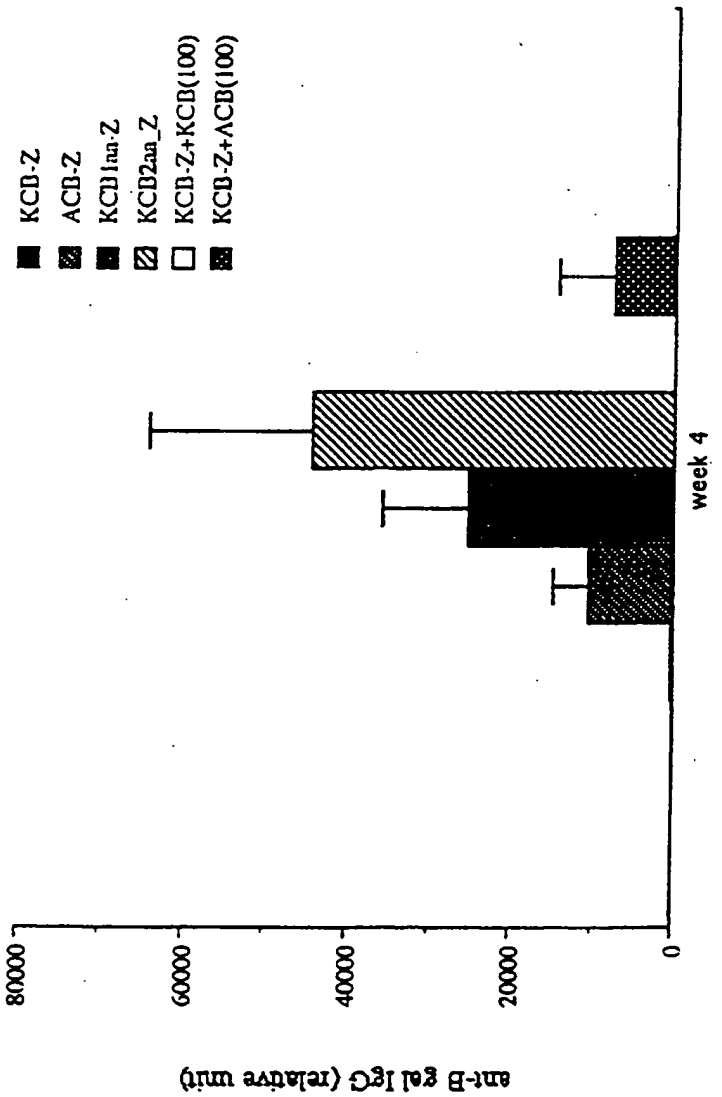

KCB-Z
ACB-Z
KCB1aa-Z
KCB2aa_Z
KCB-Z+KCB(100)
KCB-Z+ACB(100)
week 4
0
20000
40000
60000
80000
anti-B gal IgG (relative unit)

FIGURE 4

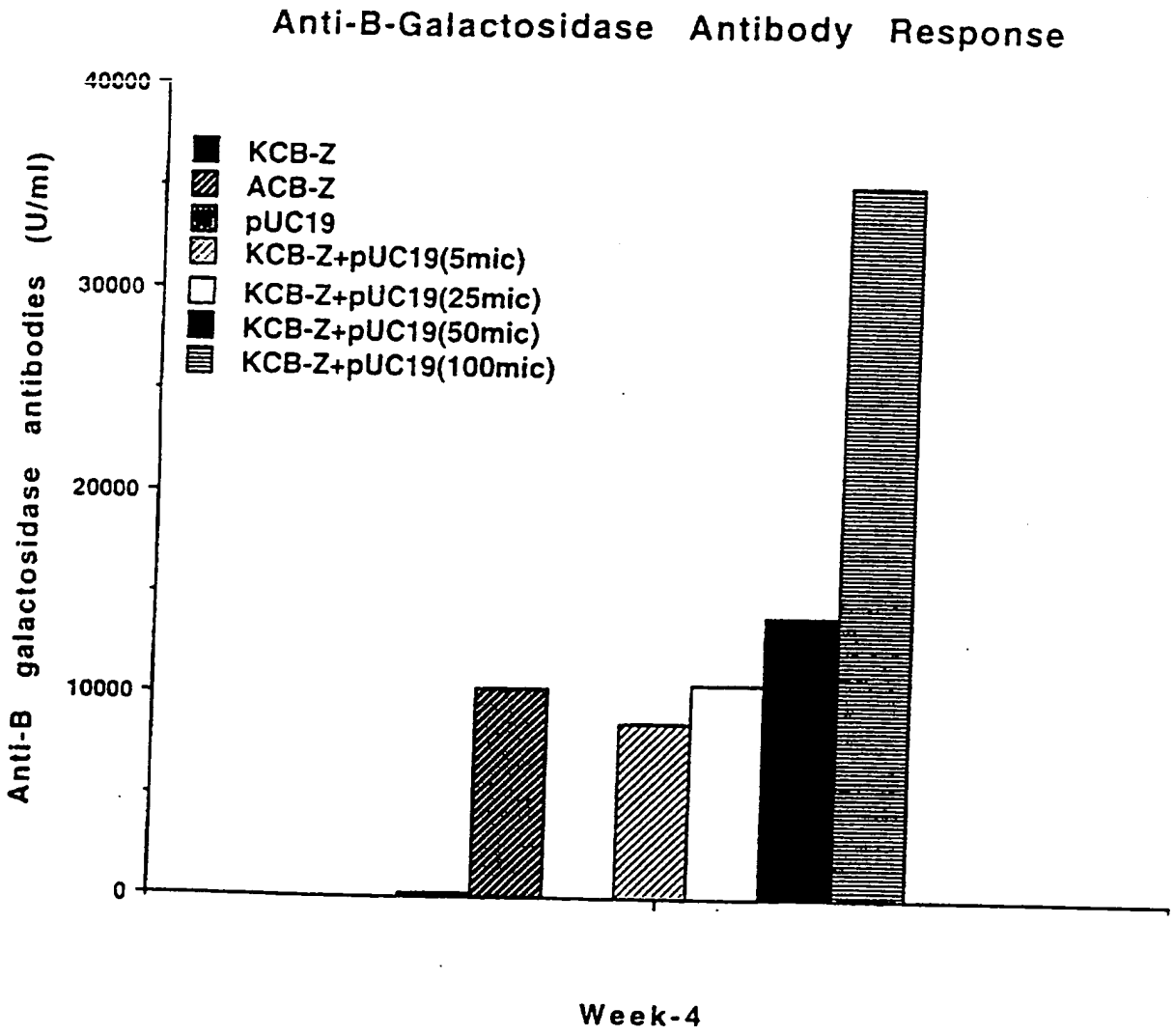

Anti-B-Galactosidase Antibody Response
Anti-B galactosidase antibodies (U/ml)
40000
30000
20000
10000
0
KCB-Z
ACB-Z
pUC19
KCB-Z+pUC19(5mic)
KCB-Z+pUC19(25mic)
KCB-Z+pUC19(50mic)
KCB-Z+pUC19(100mic)
Week-4

FIGURE 5

CTL 12/13/95-1

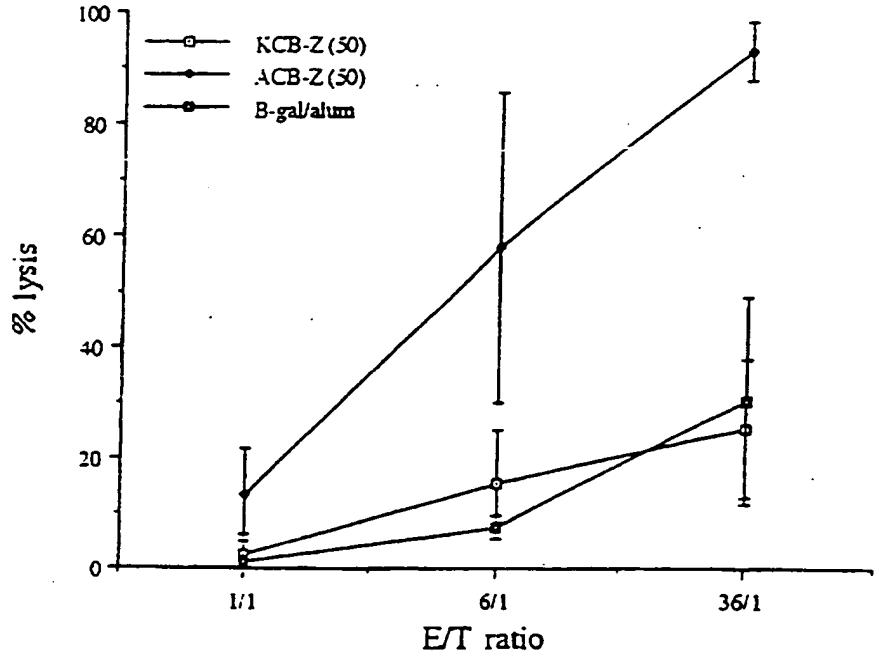


FIGURE 6

CTL 12/13/95-2

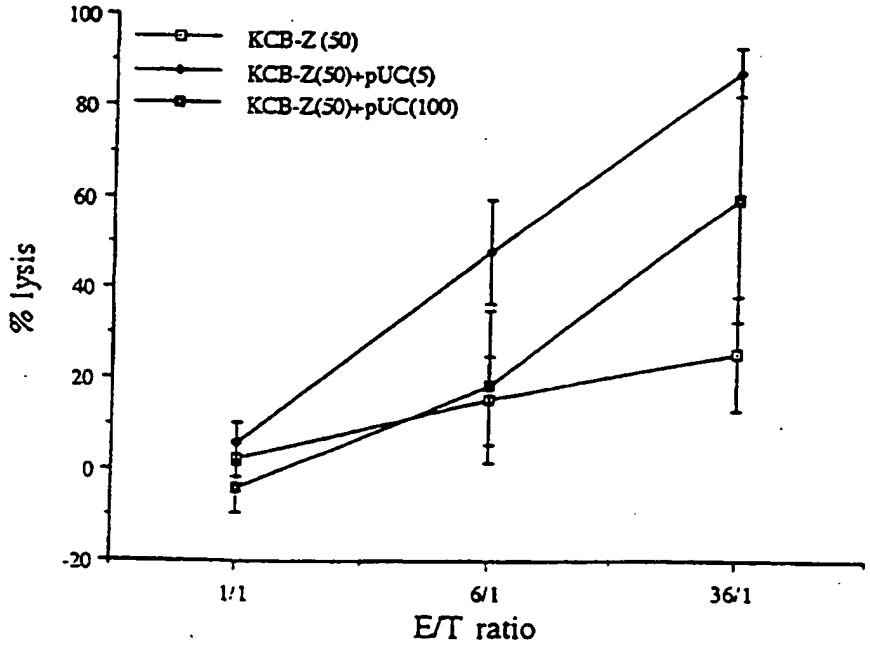


FIGURE 7

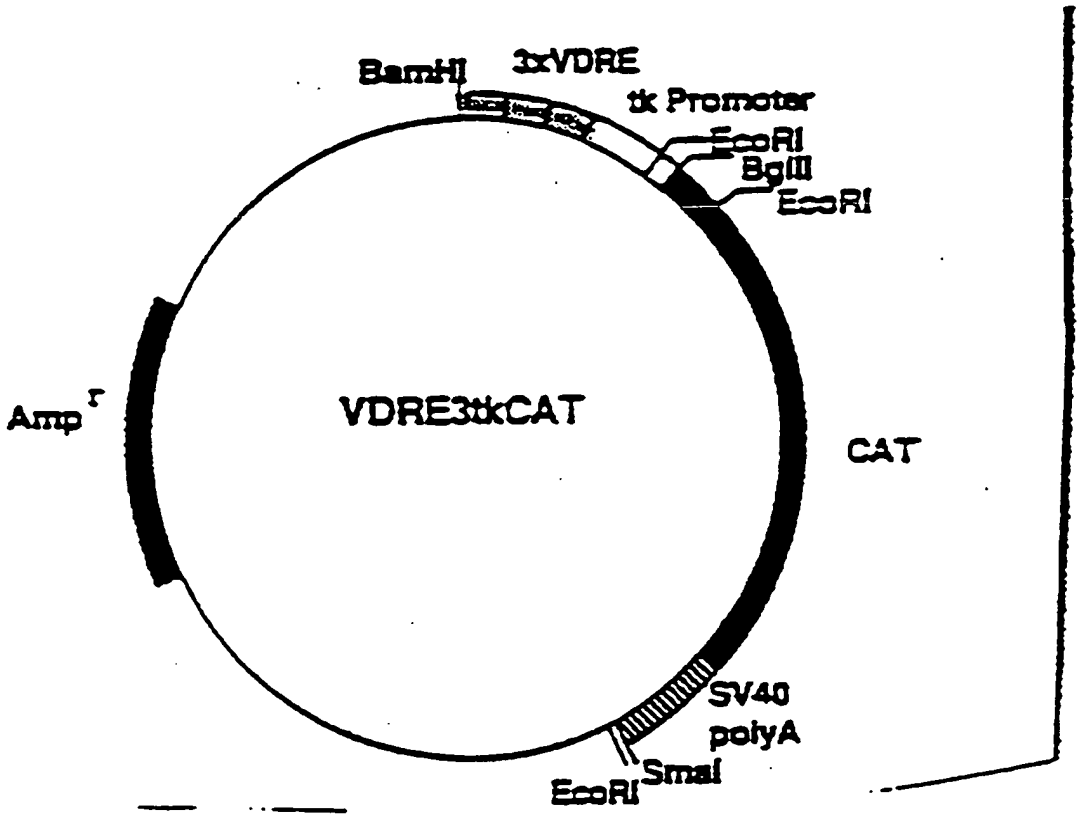

BamHI
3xVDRE
tk Promoter
EcoRI
BglII
EcoRI
Amp r
VDRE3tkCAT
CAT
SV40
polyA
SmaI
EcoRI

FIGURE 8

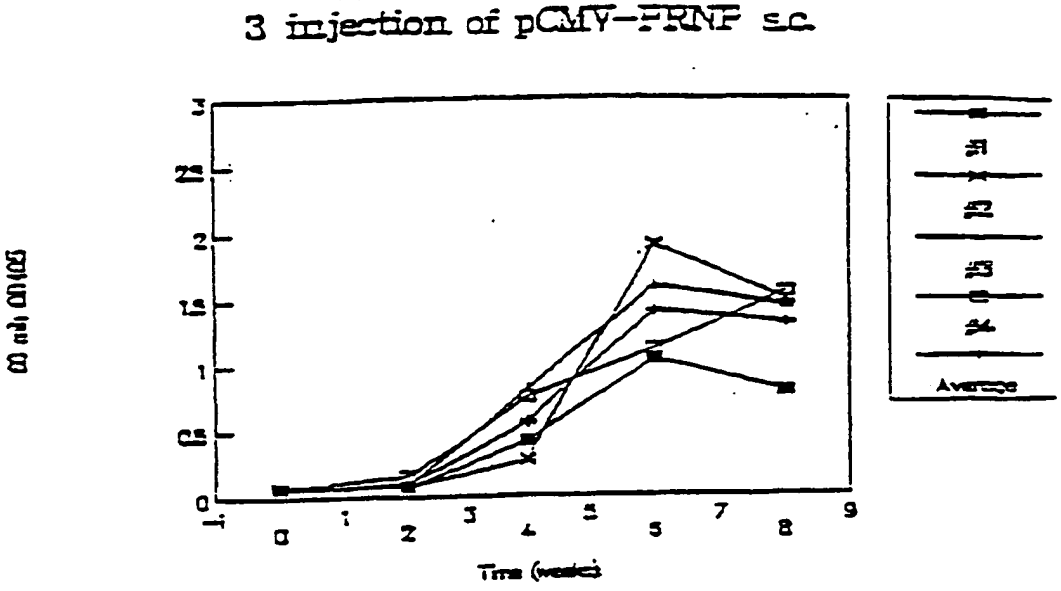


FIGURE 9a

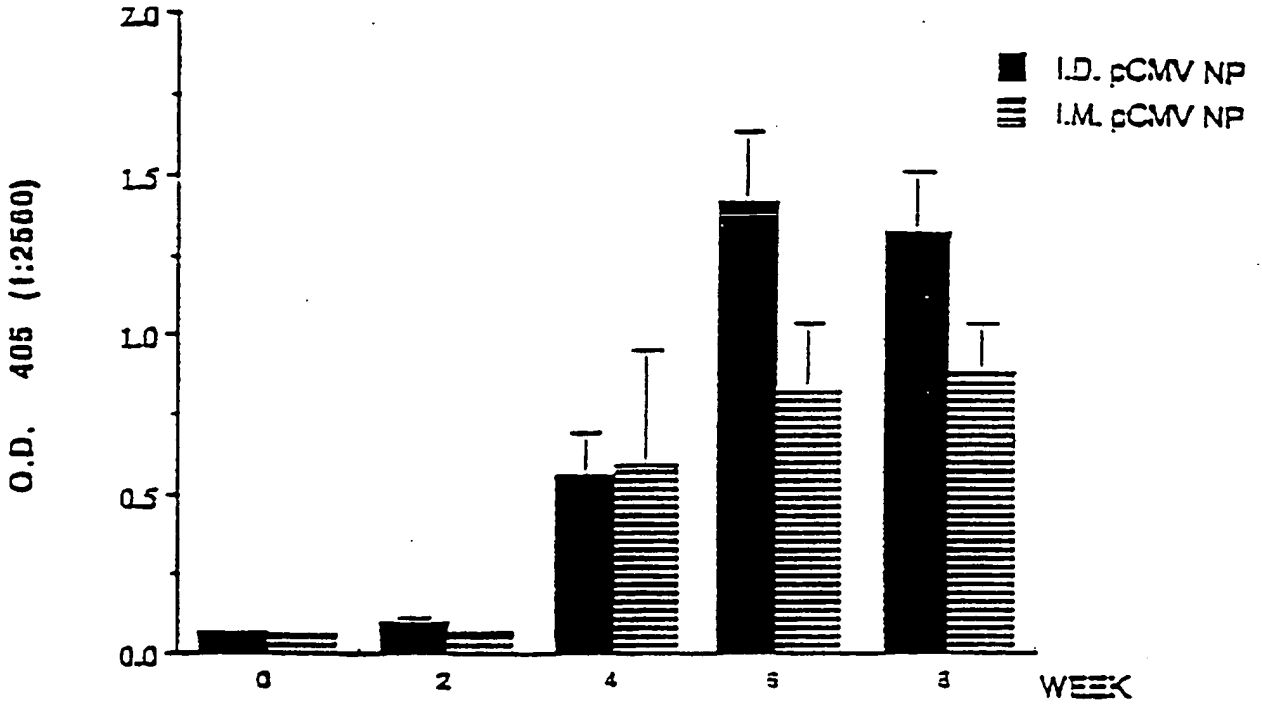

Comparison of I.D. and I.M. gene Immunization
I.D. pCMV NP
I.M. pCMV NP
O.D. 405 (1:2500)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
WEEK

FIGURE 9b

B Galactosidase Expression of Transfected Cos-1 cells

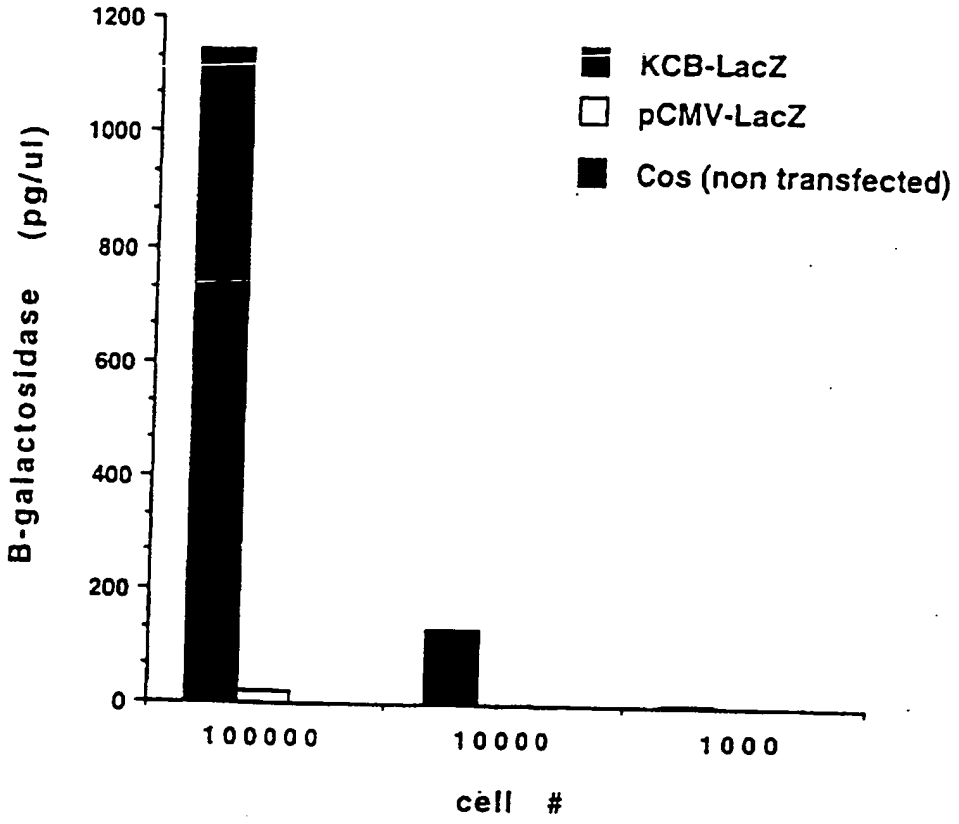


FIGURE 10

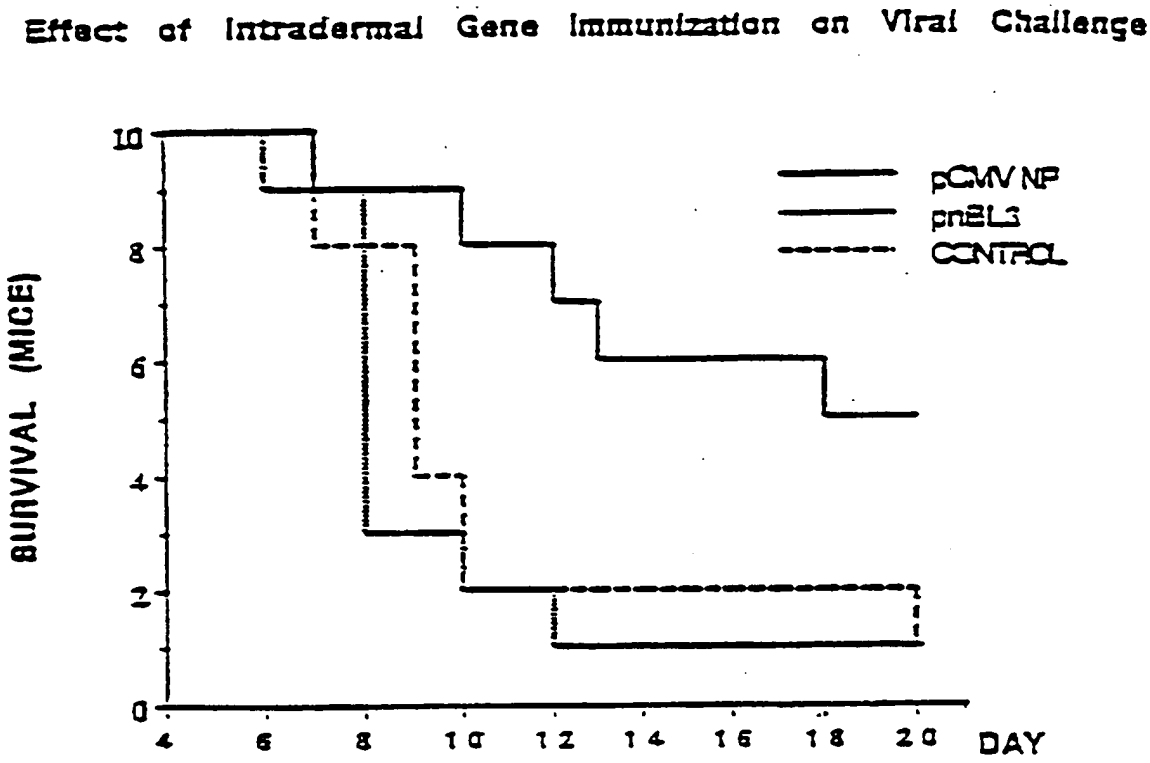

Effect of Intradermal Gene Immunization on Viral Challenge
SURVIVAL (MICE)
10
8
6
4
2
0
4
6
8
10
12
14
16
18
20
DAY
pCMV NP
pm2L3
CONTROL

FIGURE 11

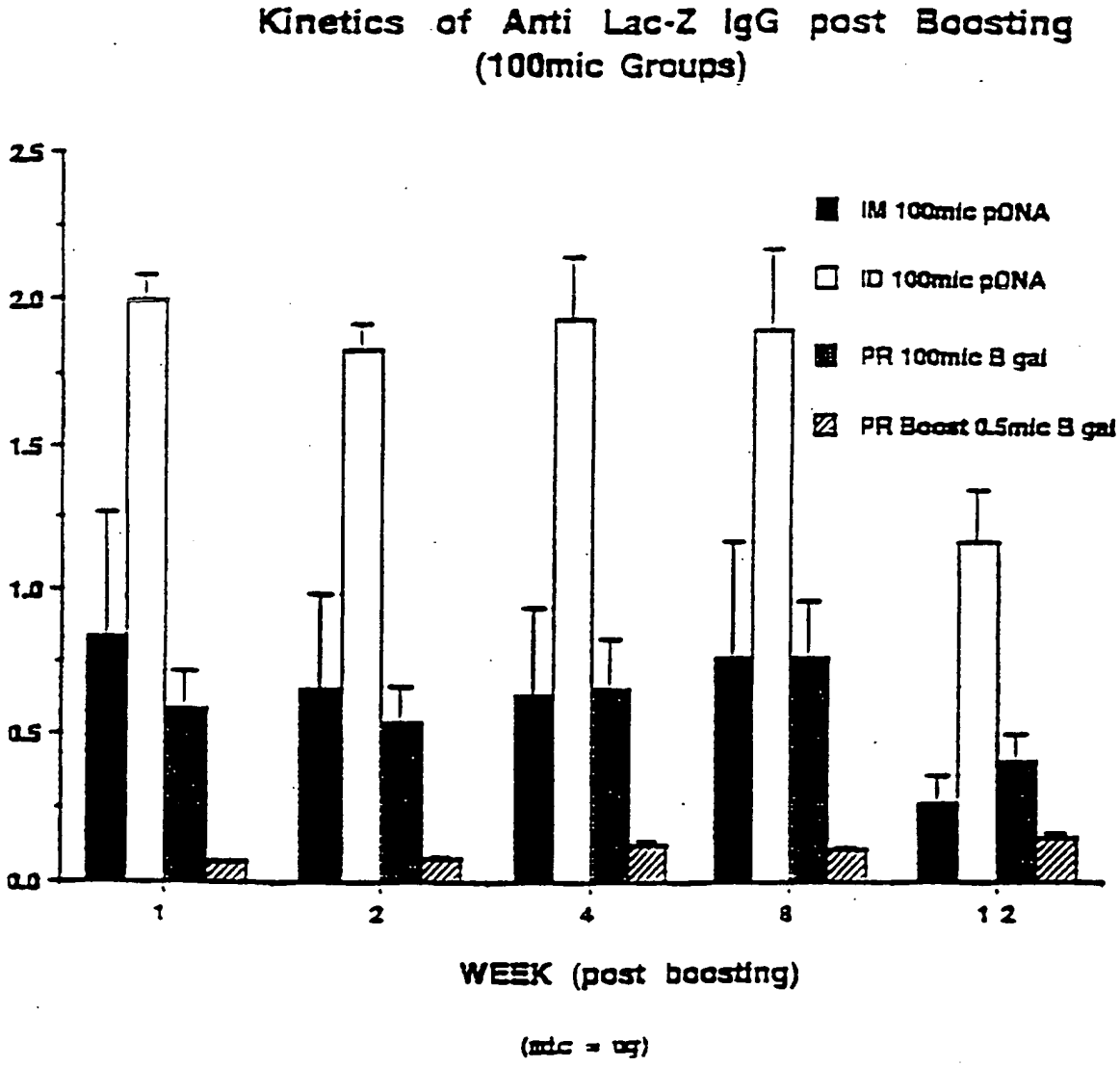

Kinetics of Anti Lac-Z IgG post Boosting
(100mic Groups)
2.5
2.0
1.5
1.0
0.5
0.0
IM 100mic pDNA
ID 100mic pDNA
PR 100mic B gal
PR Boost 0.5mic B gal
1
2
4
8
12
WEEK (post boosting)
(mic = ug)

FIGURE 12

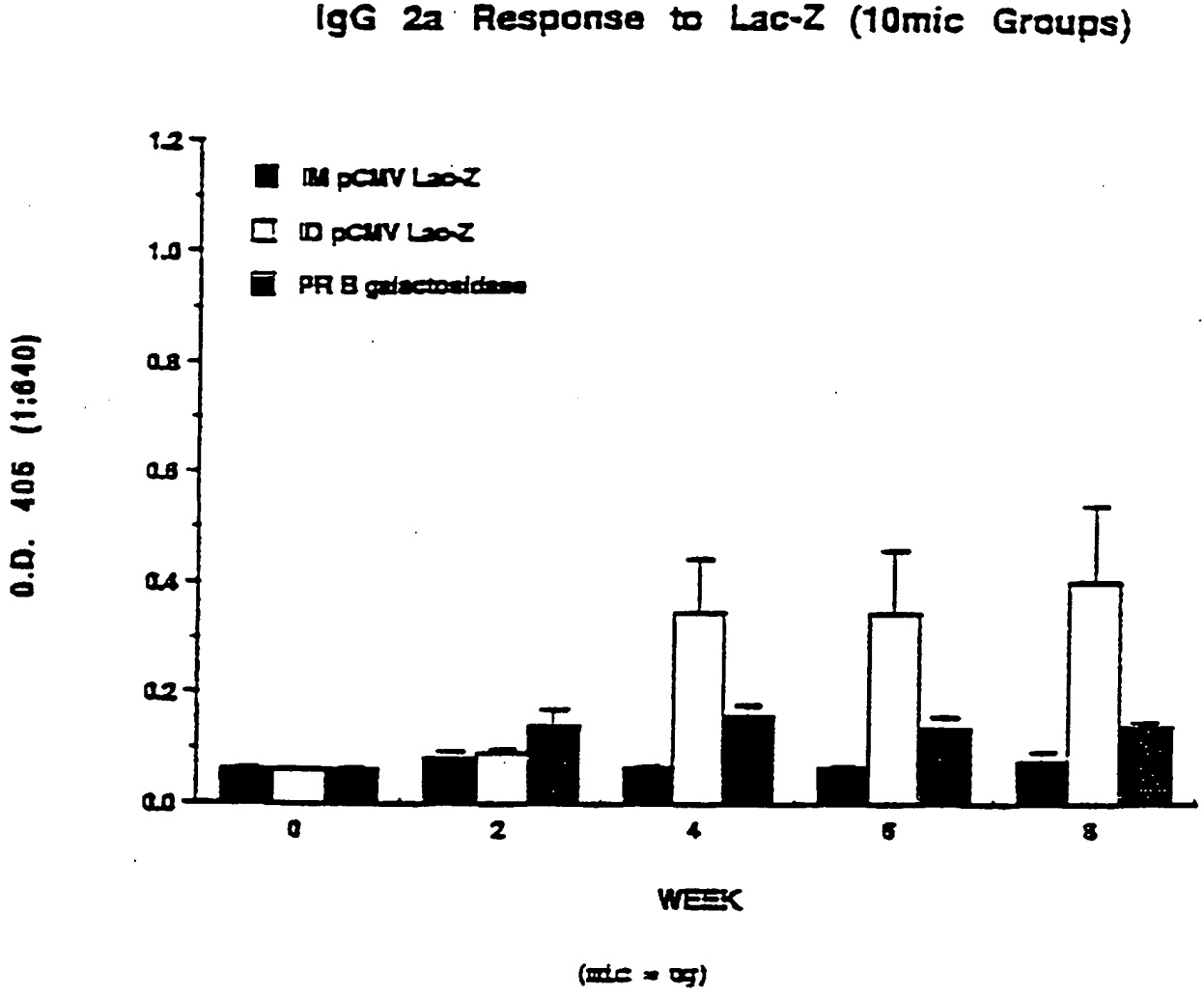

IgG 2a Response to Lac-Z (10mic Groups)
IM pCMV Lac-Z
ID pCMV Lac-Z
PR B galactosidase
O.D. 405 (1:640)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
WEEK
(mic = ug)

FIGURE 13

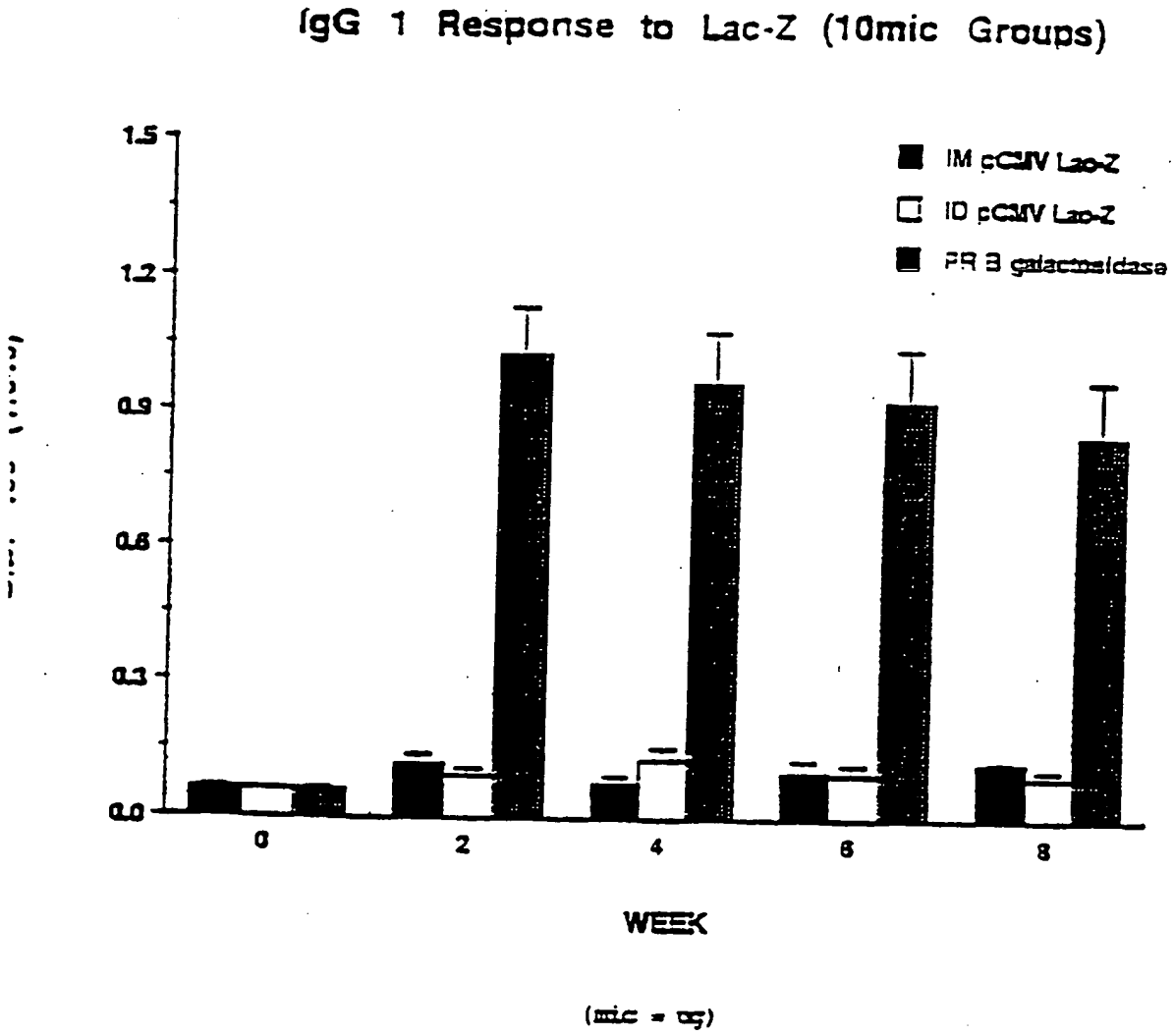

IgG 1 Response to Lac-Z (10mic Groups)
IM pCMV Lac-Z
ID pCMV Lac-Z
PR B galactosidase
1.5
1.2
0.9
0.6
0.3
0.0
0
2
4
6
8
WEEK
(mic = ug)

FIGURE 14

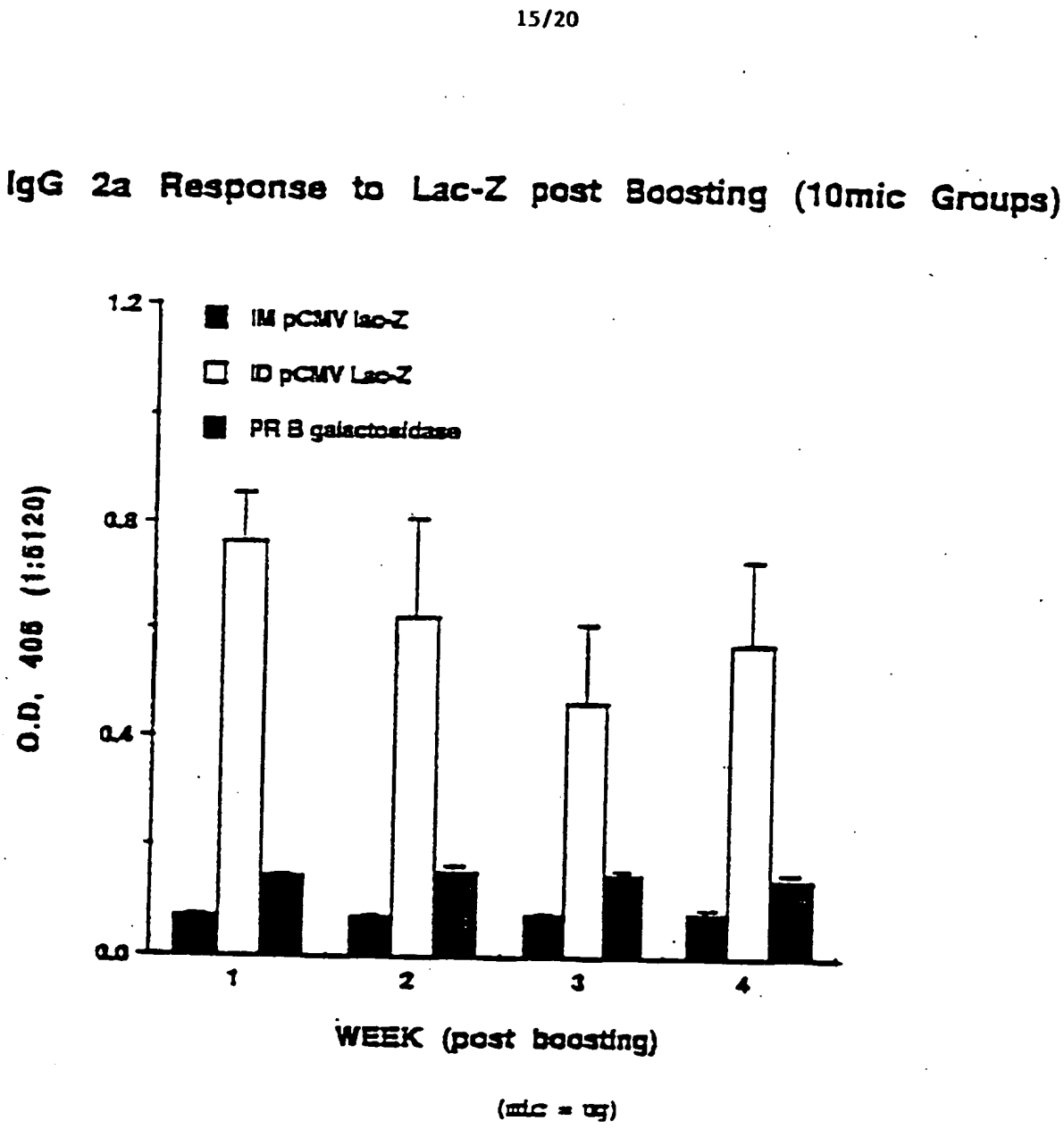

IgG 2a Response to Lac-Z post Boosting (10mic Groups)
IM pCMV lac-Z
ID pCMV Lac-Z
PR B galactosidase
O.D. 405 (1:6120)
1.2
0.8
0.4
0.0
1
2
3
4
WEEK (post boosting)
(mic = ug)

FIGURE 15

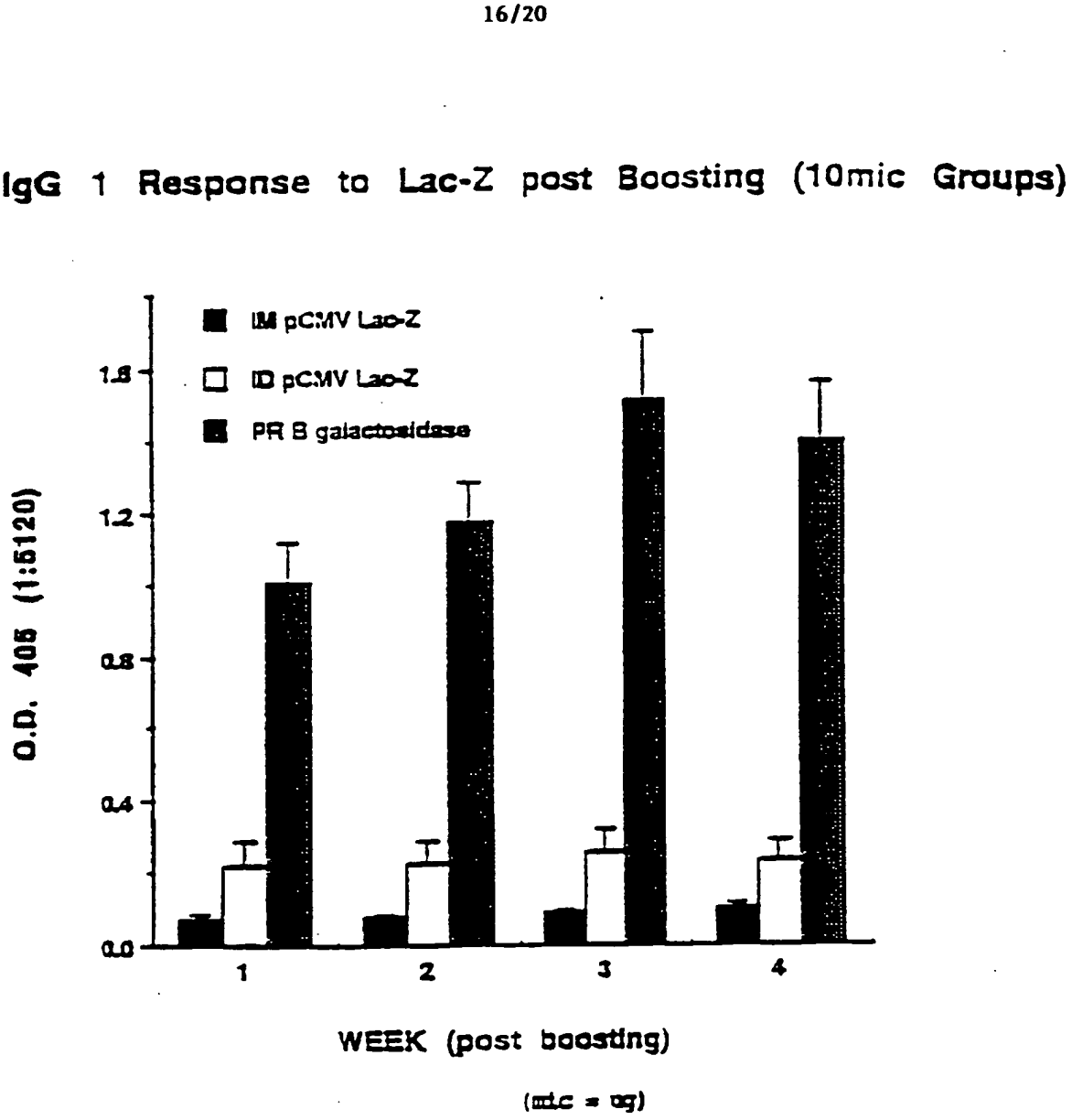

IgG 1 Response to Lac-Z post Boosting (10mic Groups)
IM pCMV Lac-Z
ID pCMV Lac-Z
PR B galactosidase
O.D. 405 (1:5120)
1.6
1.2
0.8
0.4
0.0
1
2
3
4
WEEK (post boosting)
(mic = ug)

FIGURE 16

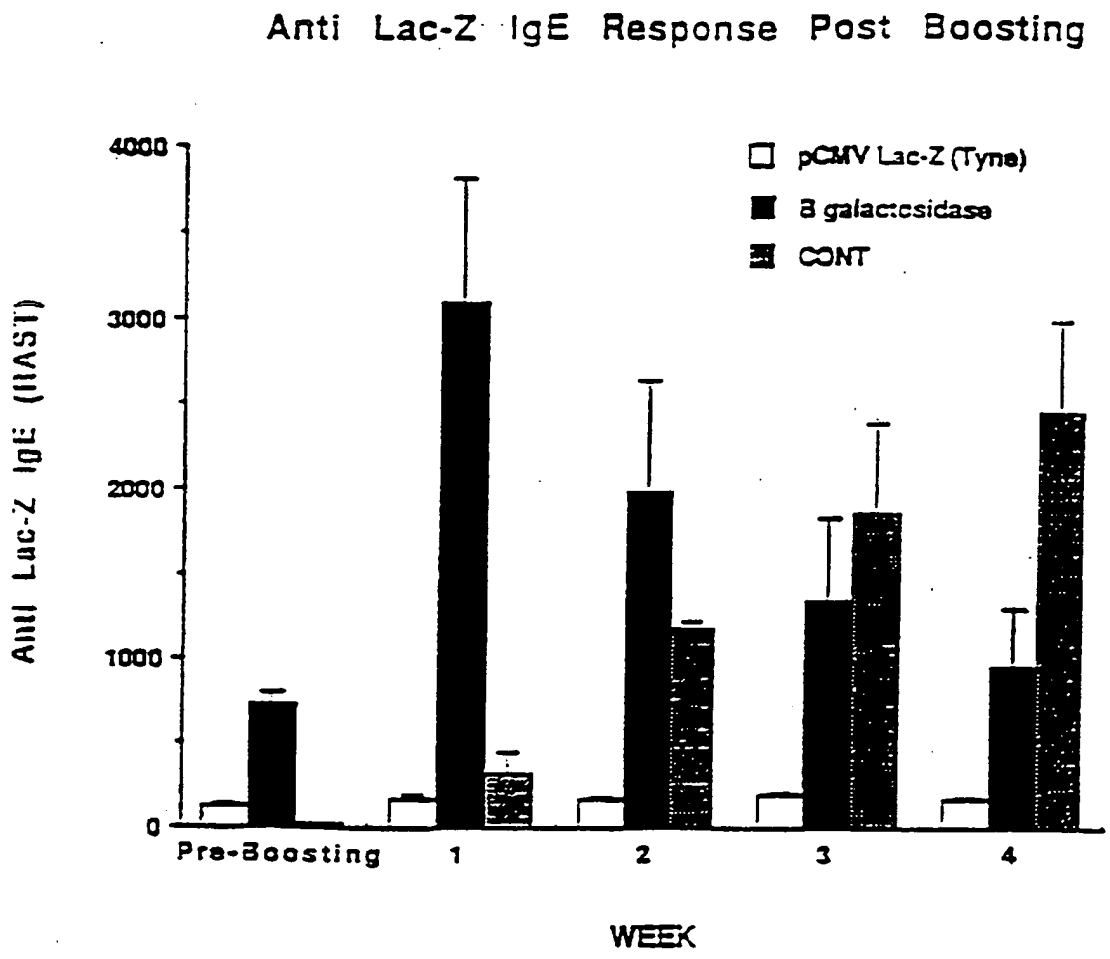

Anti Lac-Z IgE Response Post Boosting
pCMV Lac-Z (Tyne)
B galactosidase
CONT
Anti Lac-Z IgE (IIAST)
4000
3000
2000
1000
0
Pre-Boosting
1
2
3
4
WEEK

FIGURE 17

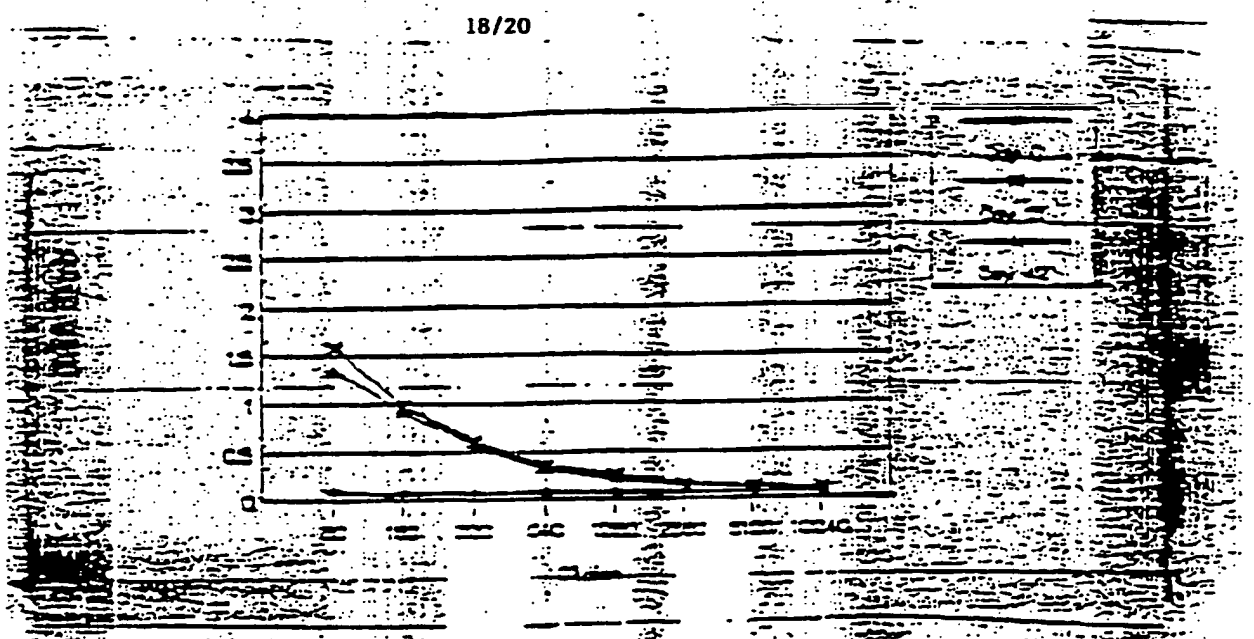


FIGURE 13

FIGURE 19

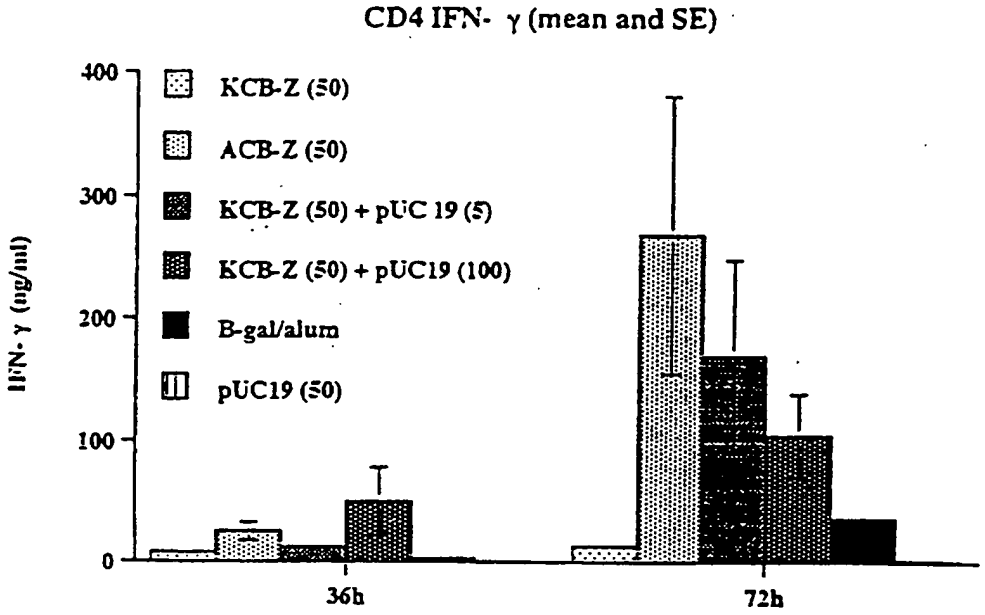
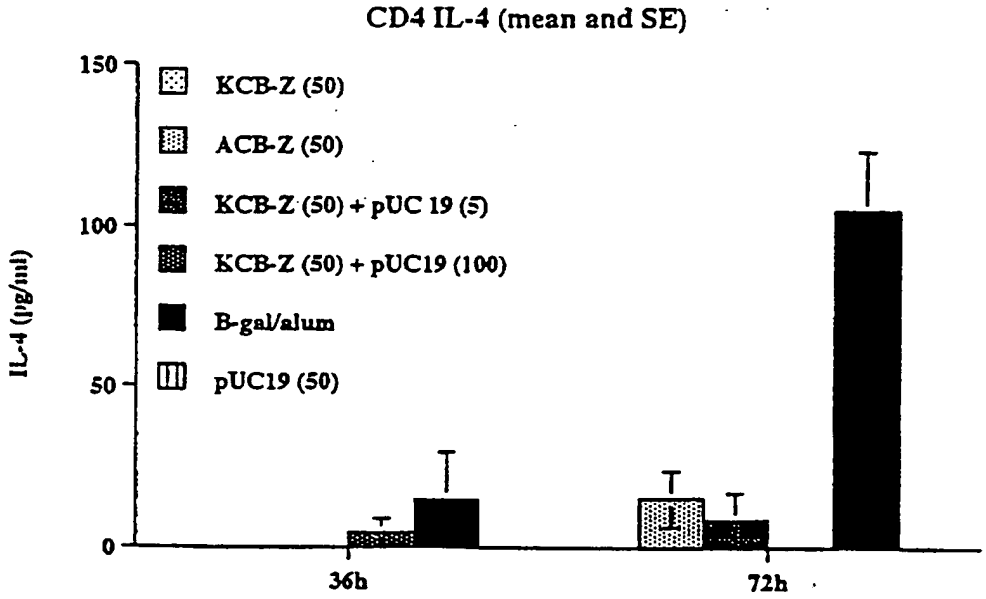

CD4 IFN- γ (mean and SE)
IFN- γ (ng/ml)
400
300
200
100
0
KCB-Z (50)
ACB-Z (50)
KCB-Z (50) + pUC 19 (5)
KCB-Z (50) + pUC19 (100)
B-gal/alum
pUC19 (50)
36h
72h

FIGURE 20


CD4 IL-4 (mean and SE)
IL-4 (pg/ml)
150
100
50
0
KCB-Z (50)
ACB-Z (50)
KCB-Z (50) + pUC 19 (5)
KCB-Z (50) + pUC19 (100)
B-gal/alum
pUC19 (50)
36h
72h

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01277

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 15/00, 15/63, 15/79, 15/09; A61K 48/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/320.1, 6, 69.1, 172.3; 514/44; 935/62, 55, 71, 65; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS, MEDLINE, CAPLUS, EMBASE, STN, APS
search terms: palindrome, vector, immune, immunostimulate, therapy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0468520 A2 (MITSUI TOATSU CHEMICALS, INC.) 29 January 1992 (29.01.92), entire patent.	1-39
Y	YAMAMOTO S. Mode of action of oligonucleotide fraction extracted from Mycobacterium bovis BCG. Kekkaku. 1994, Vol. 69, No. 9, pages 571-574, especially abstract.	1-39
Y	CARLBERG C. RXR-Independent Action of the Receptors for Thyroid Hormone Retinoid Acid and Vitamin D on Inverted Palindromes. Biochemical and Biophysical Research Communications. 30 September 1993, Vol. 195, No. 3, pages 1345-1353.	1-39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
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"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search
06 MAY 1997

Date of mailing of the international search report
04 JUN 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01277

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YAMAMOTO et al. Unique Palindromic Sequences in Synthetic Oligonucleotides are Required to Induce INF and Augment INF-Mediated Natural Killer Activity. J. of Immunology. 15 June 1992, Vol. 148, No. 12, pages 4072-4076.	1-39
Y	OHI et al. Construction and Replication of an Adeno-Associated Virus Expression Vector that Contains Human Beta-globin cDNA. Gene. 14 May 1990, Vol. 89, No. 2, pages 279-282.	1-39
Y	YAMAMOTO et al. Lipofection of Synthetic Oligodeoxyribonucleotide Having a Palindromic Sequence of AACGTT to Murine Splenocytes Enhances Interferon Production and Natural Killer Activity. Microbiol. Immunol. 1994, Vol. 38, No. 10, pages 831-836.	1-39
Y	YAMAMOTO et al. Ability of Oligonucleotides with Certain Palindromes to Induce Interferon Production and Augment Natural Killer Cell Activity Is Associated with Their Base Length. Antisense Research and Development. 1994, Vol. 4, pages 119-122.	1-39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01277

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/320.1, 6, 69.1, 172.3; 514/44; 935/62, 55, 71, 65; 536/23.1, 24.5